CONGENITAL MUSCULAR DYSTROPHY IN 2010

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2010

I, Emma Clement confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Congenital Muscular Dystrophies (CMDs) are a heterogeneous group of conditions that usually present in the first months of life with weakness and hypotonia. Extramuscular manifestations are common and may include brain, skin and eye abnormalities. CMDs are relatively rare disorders and despite the major progress made over the last 2 decades in identifying, mapping and investigating these conditions, there remains a lot to be learned. Little is known about the relative frequency of the various forms of CMD in the UK population. Experience had shown that founder mutations are common in different ethnic populations and epidemiological studies performed in other countries are of limited value in this regard. Since 2001, the Dubowitz Neuromuscular Centre (DNC) has been the National Commissioning Group UK Centre for CMD. As such we are in the privileged position to have access to a large number of UK patients with CMD. I analysed a cohort of 214 CMD referrals to the DNC between 2001 and 2008 with a view to reporting the diagnostic outcome and the frequency of the various forms of CMD encountered in our patient population.

The second part of the thesis is concerned with the dystroglycanopathies, a recently described group of CMDs associated with aberrant glycosylation of alpha dystroglycan. To date, 7 genes have been identified, some of which give rise to multiple dystroglycanopathy phenotypes. I studied the genotype-phenotype relationship in a large group of dystroglycanopathy patients, reporting new clinical phenotypes and establishing the mutation frequency in this group. I also report in detail the spectrum of MRI brain changes seen in 27 dystroglycanopathy patients.

In summary, this work reports the diagnostic outcome in the largest cohort of UK CMD cases studied and refines the genotype-phenotype correlation in patients with dystroglycanopathies.

PUBLICATIONS PERTAINING TO THE WORK WITHIN THIS THESIS

Fukutin Gene Mutations in Steroid-Responsive Limb Girdle Muscular Dystrophy.
Godfrey C, Escolar D, Brockington M, **Clement E** *et al*.
Ann Neurol 2006; 60: 603-610

Refining genotype-phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan
Godfrey C*, **Clement E***, Mein R, Brockington M *et al.*
Brain 2007; 130(10):2725-2735

Muscular dystrophies due to defective glycosylation of dystroglycan
Muntoni F, Brockington M, Godfrey C, Ackroyd M, Robb S, Manzur A, Kinali M, Mercuri E, Kaluarachchi M, Feng L, Jimenez-Mallebrera C, **Clement E**, *et al*. Acta Myologica 2007 Dec;26(3):129-35

Differential diagnosis of congenital muscular dystrophies.
Klein A, **Clement E**, Mercuri E and Muntoni F. Eur J Paediatr Neurol. 2008 Sep;12(5):371-7.

Mild POMGnT1 mutations underlie a novel Limb Girdle Muscular Dystrophy variant .
Clement E, Godfrey C, Tan J, Brockington M *et al*.
Arch Neurology 2008; 65:137-141

Brain Involvement in Muscular Dystrophies with Defective Dystroglycan Glycosylation
Clement E, Mercuri E, Godfrey C, *et al*. Ann Neurol 2008;64:573-582

A Comparative Study of alpha-Dystroglycan Glycosylation in Dystroglycanopathies Suggests that the Hypoglycosylation of alpha-Dystroglycan Does Not Consistently Correlate with Clinical Severity.
Jimenez-Mallebrera C, Torelli S, Feng L, Kim J, Godfrey C, **Clement E** *et al.*
Brain Pathol. 2009 Oct;19(4):596-611

Natural history of Ullrich congenital muscular dystrophy.
Nadeau A, Kinali M, Main M, Jimenez-Mallebrera C, Aloysius A, **Clement E** *et al.*
Neurology. 2009 Jul 7;73(1):25-31.

Muscle MRI in FHL1-linked reducing body myopathy.
Astrea G, Schessl J, **Clement E**, *et al*.
Neuromuscul Disord. 2009 Oct;19(10):689-91

Muscle Magnetic Resonance Imaging Involvement in Muscular Dystrophies with Rigidity of the Spine.
Mercuri E, **Clement E**, Offiah A, *et al* .Ann Neurol 2010 Feb;67(2):201-8

Genotype-phenotype correlation in a large population of muscular dystrophy patients with LAMA2 mutations.
Geranmayeh F, **Clement E**, Feng LH, *et al*. Neuromuscul Disord. 2010 Apr;20(4):241-50

COLLABORATORS

Clinical evaluations were carried out by myself and Professor F Muntoni at the Dubowitz Neuromuscular Centre, Institute of Child Health, University College London.

The evaluation of muscle pathology was performed by Professor C Sewry, Dr L Feng and Dr C Jimenez-Mallebrera at the Dubowitz Neuromuscular Centre, Institute of Child Health, University College London.

Mutation screening was carried out by Dr C Godfrey, Dr S Abbs, Mrs R Mein, Mr T Cullup and Miss J Pagan at Guy's DNA laboratory, GSTS Pathology, Guy's Hospital, London.

POMGNT1 enzymatic profiling was performed by Dr H Schachter, Dr J Vajsar and Dr J Tan at the Department of Structural Biology and Biochemistry, University of Toronto, Canada.

Analysis of brain MRI scans was performed in collaboration with; Professor M Rutherford at the Robert Steiner Magnetic Resonance Unit, Clinical Sciences Centre, Hammersmith Hospital, Imperial College London, Professor E Mercuri at the Department of Paediatrics, Hammersmith Hospital, London and Department of Paediatric Neurology, Rome, Italy, Professor AJ Barkovich, Department of Radiology, University of California, San Francisco, USA and Professor F Muntoni at the Dubowitz Neuromuscular Centre, Institute of Child Health, University College London.

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ABBREVIATIONS

AAV	Adeno associated viral
AD	Autosomal dominant
ADG	Alpha dystroglycan
AR	Autosomal recessive
BFPP	Bilateral frontoparietal polymicrogyria
BP	Base pair
BM	Bethlem Myopathy
CDG	Congenital disorder of glycosylation
CK	Creatine Kinase
CMD	Congenital muscular dystrophy
CMD-CRB	CMD with cerebellar involvement
CMD-MR	CMD with mental retardation
CMD-no MR	CMD with no mental retardation
CNS	Central Nervous System
CP	Cortical Plate
CSA	Cyclosporin A
DAG	Dystrophin-associated glycoproteins
DGC	Dystrophin glycoprotein complex
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
DNC	Dubowitz Neuromuscular Centre
ECM	Extracellular Matrix
EDMD2	Emery Dreifuss muscular dystrophy type 2
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FCMD	Fukuyama CMD
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
INM	Inner nuclear membrane
kb	Kilobase
kDa	Kilodalton
LCMV	Lymphocytic choriomeningitis virus

LGMD	Limb girdle muscular dystrophy
LGMD2I	Limb girdle muscular dystrophy type 2I
LGMD-MR	LGMD with mental retardation
LGMD-no MR	LGMD with no mental retardation
MDC1A	Muscular dystrophy congenital type 1A
MDC1B	Muscular dystrophy congenital type 1B
MDC1C	Muscular dystrophy congenital type 1C
MDC1D	Muscular dystrophy congenital type 1D
MEB	Muscle eye brain disease
mPTP	Mitochondrial permeability transition pore
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MZ	Marginal Zone
NAC	*N*-acetylcysteine
NCG	National Commissioning Group
NSCAG	National Specialist Committee Advisory Group
OMIM	Online Mendelian Inheritance in Man
ONM	Outer nuclear membrane
PCR	Polymerase chain reaction
PH	Periventricular heterotopia
PMG	Polymicrogyria
RNA	Ribonucleic acid
RSMD1	Rigid spine muscular dystrophy type 1
SP	Subplate
UCMD	Ullrich congenital muscular dystrophy
VZ	Ventricular zone
WWS	Walker-Warburg syndrome

AIMS OF THIS THESIS

Despite the progress made over the last two decades and the increasing number of investigative options available, congenital muscular dystrophies (CMD) are still an emerging group of disorders and much remains to be learnt about the phenotypic heterogeneity and molecular basis of these conditions

The aims of this thesis are:

1. To review the general activity of the CMD National Commissioning Group service from 2001 to 2008. To establish the relative frequency of the various forms of CMD seen within our population group and the common differential diagnoses.
2. To review our cohort of dystroglycanopathy patients.
    a. To report phenotypic and genetic heterogeneity
    b. To establish the extent and variability of brain involvement.

This thesis is divided in to 3 sections, chapters 2, 3 and 4, reflecting the main areas described above. Chapter 1 is a general introduction to CMD and relevant topics are expanded further in specific chapter introductions. Chapter 5 serves as a concluding discussion.

CHAPTER 1. INTRODUCTION

1. INTRODUCTION

1.1 CONGENITAL MUSCULAR DYSTROPHY IN 2010

Congenital muscular dystrophies (CMDs) are a heterogeneous group of mainly autosomal recessive disorders associated with dystrophic changes on muscle biopsy. These conditions present at birth or within the first few months with muscular weakness, hypotonia, delayed motor milestones and variable joint contractures. In addition, a significant proportion of affected individuals exhibit mental retardation, often with structural changes visible on Magnetic Resonance (MRI) brain imaging. Other features found in specific types include spinal rigidity, joint laxity, muscle hypertrophy, respiratory involvement and structural eye defects. Approximately a dozen different CMD phenotypes have been described to date with an associated spectrum of severity and distinct clinical features (1, 2).

CMD was first described as a disease entity over a century ago by Batten (3), however, scientific progress in this field was relatively modest until the 1990's. This decade marked a turning point during which rapid advances in understanding the clinical and molecular aspects of CMD were made in large part aided by the establishment of European Neuromuscular Centre Consortium workshops (2, 4-8).

The classification of CMDs has historically been problematic. The original phenotypic descriptions were largely based on clinical features and geographical origin of the various conditions. The first attempt to formally classify the CMDs divided them into 'classical' CMD, without intellectual impairment or overt CNS changes and those cases with clear CNS involvement (5). The discovery of abnormal merosin staining in skeletal muscle in 1994 and the subsequent reports of mutations in *LAMA2* in patients with MDC1A in 1995 paved the way for a new classification of CMD based on biochemical and molecular abnormalities. The renewed interest in this area resulted in the identification of a number of new CMD phenotypes and associated genes and also an understanding of the molecular basis of these conditions (9, 10).

The Dubowitz Neuromuscular Centre (DNC) is the designated National Commissioning Group (NCG) UK centre for CMD (previously known as NSCAG, National Specialist

Committee Advisory Group) and has a long standing clinical and academic interest in this field. As such, we are in an excellent position to collect and study genetic, clinical and pathological data from patients with CMD. In addition we have an ethically approved project covering research into this area and have recently established a biobank facility for obtaining and storing pathological specimens from disease and control patients.

1.2 SKELETAL MUSCLE STRUCTURE AND FUNCTION

Skeletal muscle varies in shape and size according to function. Each muscle is surrounded by a connective sheath known as the epimysium that is continuous with the tendon and subdivided into fascicles by the perimysium. Within each fascicle individual muscle fibres are found tightly packed and are separated by endomysium, comprised of a collagen network and extracellular matrix proteins. Individual muscle fibres (myofibres) are multinucleated and formed by the fusion of immature myoblasts (Figure 1) (4) .

Myofibres are seen in two major physiologically and histologically distinct forms. Type 1 fibres are slow twitch fibres and contain a higher quantity of mitochondria and lipid. They are suited for sustained low grade activity. Type 2 fibres are fast twitch fibres and are smaller, with more glycogen but less mitochondria and lipid. Their function is to support brief duration high intensity activity. Type 1 and 2 fibres are found randomly distributed in muscle and are seen as a chequer board pattern on cross section.

Myofibres contain sarcoplasm, enclosed by the sarcolemma. The sarcoplasm contains the sarcomeres, the contractile elements of the cell. Sarcomeres comprise actinin, which forms the Z band, and actin and myosin which are major constituents of the thin and thick filaments respectively. Surrounding the sarcomeres is the myofibrillar network, made up of several components including mitochondria, lipid, glycogen and sarcoplasmic reticulum. Surrounding the sarcolemma is the basement membrane which abuts the extracellular matrix (Figure 1).

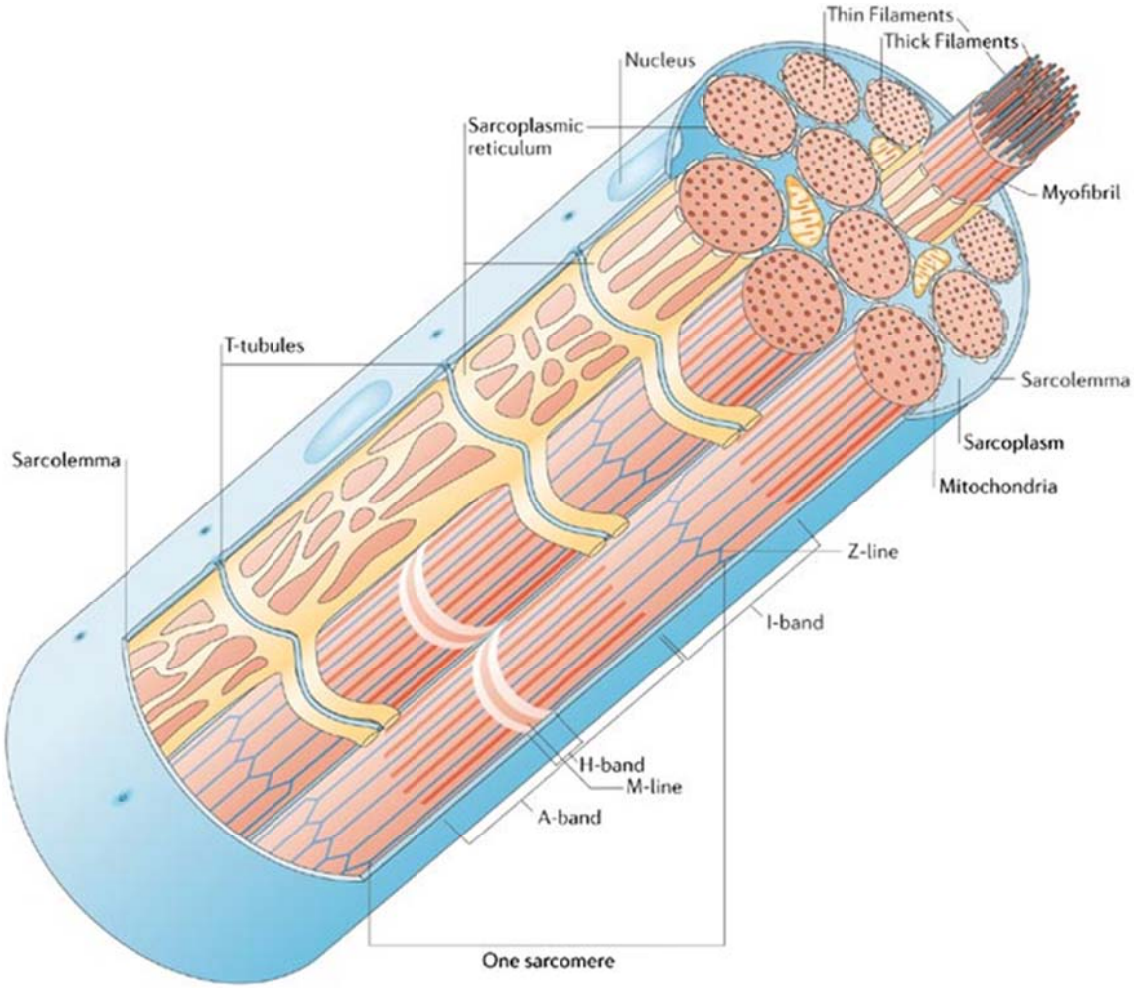


Figure 1. Skeletal Muscle Structure.

Skeletal muscle is the muscle attached to the skeleton. Hundreds of muscle fibres bundle together to make up an individual skeletal muscle. Muscle fibres (myofibres) are long, cylindrical structures that are bound by a plasma membrane (the sarcolemma) and an overlying basal lamina and when grouped into bundles (fascicles) they make up muscle. The sarcolemma forms a physical barrier against the external environment and also mediates signals between the exterior and the muscle cell.

The sarcoplasm is the specialized cytoplasm of the striated-muscle fibre that contains the usual subcellular elements along with the Golgi apparatus, abundant myofibrils, a modified endoplasmic reticulum known as the sarcoplasmic reticulum (SR), glycogen granules, myoglobin and mitochondria. Transverse (T)-tubules invaginate the sarcolemma, which allows action-potential signals to penetrate the cell and activate the

SR. As shown in the figure, the SR forms a network around the myofibrils, storing and providing the Ca^{2+} that is required for muscle contraction.

Myofibrils are contractile units that consist of an ordered arrangement of longitudinal myofilaments. Myofilaments can be either thick filaments (comprised of myosin) or thin filaments (comprised of actin). Together they produce movement by contraction through the sliding filament model.

Protein assemblies known as costameres, located on the peripheral myofibrils of the myofibre, line up with the Z-disk. Costameres physically link the sarcomeres, which produce force through contraction, with the sarcolemma and, are proposed to transmit this force across the sarcolemma to the extracellular matrix and on to neighbouring muscle cells. So, during both contraction and relaxation, sarcomere length remains consistent between muscle cells within skeletal muscle.

Many proteins known to be defective in the CMDs are components of the basement membrane or extracellular matrix. The basement membrane is comprised of the outer reticular layer and inner basal lamina. The outer reticular layer is fibrillar and contains collagen 6 myofibrils embedded in a proteoglycan rich substance. The inner basal lamina is linked directly to the sarcolemma and is comprised of networks of non fibrillar collagen VI and laminin linked by non-collagenous glycoproteins (4, 12). The basement membrane has a number of important functions. It has long been known that it offers the cells structural support but more recently it has been shown to have a role in developmental and regenerative processes including myogenesis and synaptogenesis. In addition it is now appreciated that it has a critical role in cell signalling through its complex network interactions with other proteins and receptors including the dystrophin-glycoprotein complex (DGC)(13, 14). The DGC is a multimeric transmembrane protein complex comprised of 3 subcomplexes; the central dystroglycan complex, the intracellular dystrophin complex and the sarcoglycan-sarcospan complex. The DGC provides a structural link between the cytoskeleton and the proteins of the basement membrane through interaction with extracellular ligands including perlecan and laminin. It is thought to protect the sarcolemma from mechanical stress during muscle contraction (Figure 2) (14) .

The nuclear envelope consists of an inner nuclear membrane (INM) and an outer nuclear membrane (ONM) separated by a perinuclear space. Nuclear pore complexes span the 2 membranes providing a link between the cytoplasm and nucleus (15, 16). The ONM is continuous with the endoplasmic reticulum. At the nuclear face of the INM lies the nuclear lamina, comprised largely of A-type and B-type lamins that form intermediate filaments providing major structural and mechanical support to the nucleus(17). In mammalian somatic cells, the predominant A-type lamins are lamin A and lamin C encoded by LMNA (18). Nesprin-1 is an actin binding protein and integral part of the ONM. It forms part of the LINC (linker of nucleoskeleton and cytoskeleton) complex that connects the actin cytoskeleton via nesprin proteins to the nuclear lamins and other components of the nuclear interior (19).

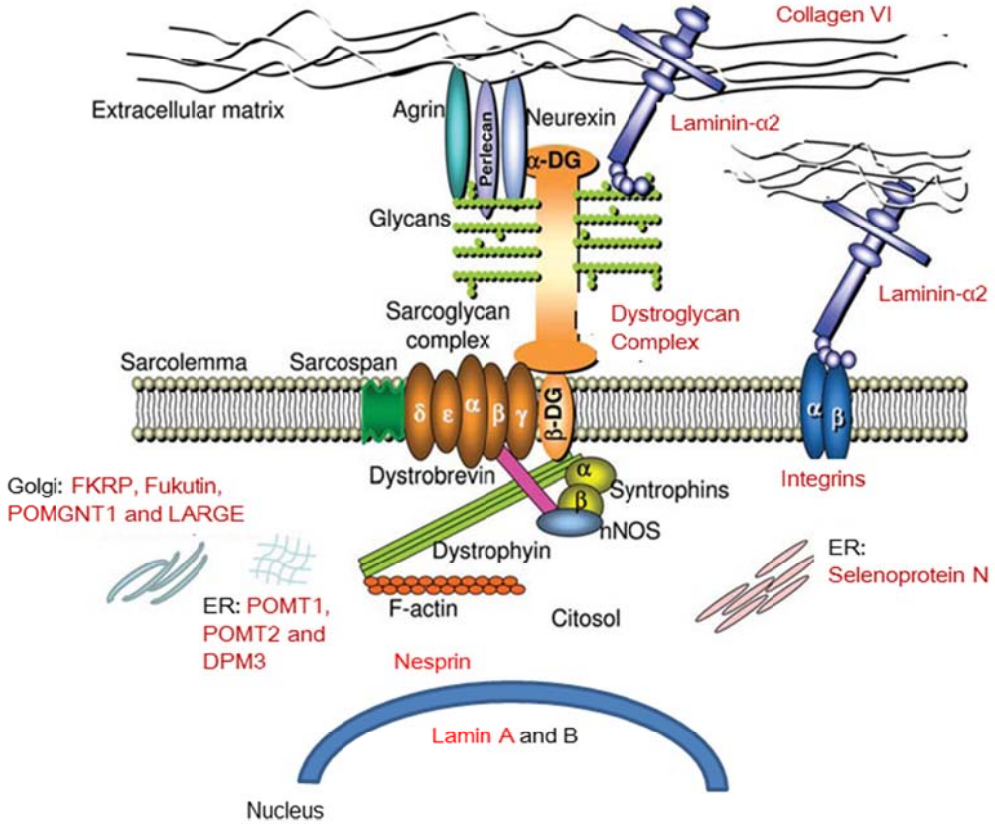


Figure 2. Diagrammatic representation of the subcellular localisation of CMD proteins within skeletal muscle.

Proteins highlighted in red are known to cause various forms of congenital muscular dystrophies.

ER; endoplasmic reticulum, POMT1; Protein-O-mannosyl transferase 1, POMT2; Protein-O-mannosyl transferase 2, POMGNT1; Protein-O-mannose 1,2-N-acetylglucosaminyltransferase 1, Fukutin-related protein; FKRP, LARGE Like-glycosyltransferase; LARGE, Dolichyl-phosphate mannosyltransferase polypeptide 3; DPM3.

Figure modified from Reed, Umbertina Conti. Congenital muscular dystrophy. Part I: a review of phenotypical and diagnostic aspects. *Arq. Neuro-Psiquiatr.* 2009, vol.67 (20) and Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1772, Matthew

T. Lisi, Ronald D. Cohn, Congenital muscular dystrophies: New aspects of an expanding group of disorders, copyright (2007), with permission (21).

1.3 CLASSIFICATION OF CMD

CMDs may be grouped according to those that occur as a result of abnormalities in external membrane, basal lamina and extracellular matrix proteins (including integrins, Laminin α2, and Collagen VI) and those CMDs resulting from abnormal glycosylation of alpha dystroglycan (ADG) secondary to mutations in known/ putative enzymes involved in this pathway (Protein-O-mannosyl transferase 1, Protein-O-mannosyl transferase 2, Protein-O-mannose 1,2-N-acetylglucosaminyltransferase 1, Fukutin, Fukutin-related protein, LARGE and Dolichyl-phosphate mannosyltransferase polypeptide 3). In addition, CMD may result from mutations in *SEPN1*, a gene that encodes an endoplasmic reticulum protein of unknown function or, more rarely, by mutations in the nuclear envelope proteins Lamin A/C and Nesprin(12) (22). The cellular location of a number of these proteins is represented in Figure 2.

The known genetic variants of CMD are summarised in table 1 and table 2 (2) (23, 24).

Functional Classification	Protein	Gene	Gene ID≠	Chromosomal Location	Disease	OMIM Number±
Structural protein of basement membrane, extracellular matrix or external membrane protein	Laminin α 2	*LAMA2*	156225	6q2	MDC1A	607855
	Collagen VI	*Col6A1* *Col6A2* *Col6A3*	120220 120240 120250	21q2 21q2 2q3	UCMD BM	254090 158810
	Integrin α 7	*ITGA7*	600536	12q	Integrin α 7 deficiency	600536
Dystroglycanopathy	Protein-O-mannosyltransferase 1	*POMT1*	607423	9q34	WWS*	236670
	Protein-O-mannosyltransferase 2	*POMT2*	607439	14q24	WWS*	236670
	Protein-O-linked mannose beta 1,2-N-acetylglucosaminyltransferase	*POMGNT1*	606822	1p34	MEB*	253280
	Fukutin	*FKTN*	607440	9q31	FCMD*	253800
	Fukutin Related Protein	*FKRP*	606596	19q13	MDC1C*	606612
	Like-Glycosyltransferase	*LARGE*	603590	22q12	MDC1D*	608840
	Dolichyl-phosphate mannosyltransferase polypeptide 3	*DPM3*	605951	1q22	CDG1o¥	612937
Endoplasmic reticulum protein	Selenoprotein N,1	*SEPN1*	606210	1p35-36	RSMD1†	606210
Nuclear envelope protein	Lamin A/C	*LMNA*	150330	1q21	EDMD2‡	181350
	Nesprin	*SYNE1*	608441	6q25	CMD with adducted thumbs(25)	

Table 1. Congenital Muscular Dystrophy Variants with known gene defects.

≠ Entrez Gene Identifier from the NCBI database (http://www.ncbi.nlm.nih.gov/).[±] Online Mendelian Inheritance in Man reference (http://www.ncbi.nlm.nih.gov/OMIM). *Denotes original phenotype described in association with gene, the dystroglycanopathies show considerable genetic heterogeneity.†SEPN1 mutations may also be associated with other myopathy phenotypes (see section 1.6.3). ‡LMNA mutations are associated with a number of other non-CMD phenotypes (see section 1.6.4). ¥ To date mutations in DPM3 have only been detected in a single patient.

MDC1A; Congenital Muscular Dystrophy type 1A, UCMD; Ullrich Congenital Muscular Dystrophy, BM; Bethlem Myopathy, WWS; Walker Warburg Syndrome, MEB; Muscle Eye Brain Disease, FCMD; Fukuyama CMD, MDC1C; Congenital Muscular Dystrophy type 1C, MDC1D; Congenital Muscular Dystrophy type 1D, CGD1o; Congenital Disorder of Glycosylation type 1o, RSMD1; Rigid Spine Muscular Dystrophy, EDMD2; Autosomal Dominant Emery Dreifuss Muscular Dystrophy.

Disease	OMIM Number±	Chromosomal Location	Phenotype	Reference
Congenital Muscular Dystrophy with Joint Hyperlaxity (CMDH)	-	3p23†	CMD with joint hyperlaxity	Tetreault *et al*.(26)
Congenital Muscular Dystrophy associated with 4p16.3	609456	4p16.3	Merosin positive CMD*	Sellick *et al*.(27)
Congenital Muscular Dystrophy type 1B (MDC1B)	604801	1q42	CMD with early respiratory failure	Brockington *et al*.(28)

Table 2. Congenital Muscular Dystrophy loci, gene defect unknown in 2006.

*subsequently shown to be a congenital myasthenia resulting from mutations in DOK7. (Francesco Muntoni personal communication)

†subsequently shown to be due to mutations in integrin α9(29)

.± Online Mendelian Inheritance in Man reference (*http://www.ncbi.nlm.nih.gov/OMIM*).

1.4 CLINICAL FEATURES OF CMD

By definition, the muscular system is always affected in CMD although the degree to which it is affected is very variable. To a large degree it is dependent on the type of CMD a patient has, although variability within phenotypic groups may also be seen. The main musculoskeletal features are weakness, hypotonia, muscle wasting or hypertrophy. Some patients are severely affected and may be recognised prenatally with decreased foetal movement. This is typical in cases of WWS. Others are clearly hypotonic and weak at birth with minimal or reduced movement, commonly seen in cases of MDC1A, MDC1C and MEB. Others may appear relatively normal from a musculoskeletal perspective in the first few weeks, with weakness and failure to achieve motor milestones recognised outside the neonatal period. This would be expected in milder cases of UCMD, BM and also in the some dystroglycanopathy phenotypes.

Maximal motor achievement is equally variable. In some forms of CMD, for example WWS, motor progress may be virtually absent. Others progress to sitting with or without support but would not be expected to achieve independent ambulation. This would be typical of MDC1A or MDC1C. At the milder end of the spectrum patients with milder UCMD, BM and SEPN1 related myopathy would be expected to achieve ambulation and in the case of BM and SEPN1 related myopathy would anticipate remaining ambulant into the third decade and maybe beyond. The progression of weakness in CMD is usually relatively static with increasing disability often the result of contractures and scoliosis.

Individuals with CMD may also have other musculoskeletal manifestations including contractures, congenital dislocation of the hips, torticollis, spinal rigidity and scoliosis. The presence of such features may be important diagnostically; patients with UCMD for example frequently have one or more of these features present at birth. Weakness can also extend to respiratory musculature. In RSMD1 and MDC1B diaphragmatic weakness causes respiratory insufficiency early in the disease course. Respiratory involvement is also invariable in UCMD and MDC1A, whilst in other forms of CMD it does not appear to cause significant early morbidity.

Cardiac involvement in CMD is unusual with a few exceptions. Patients with congenital onset EDMD2 may have conduction defects including paroxysmal atrial tachycardia and ventricular tachycardia. Sudden death has also been reported.(30) Cardiac involvement is also seen in dystroglycanopathy patients with *FKTN* and *FKRP*

mutations and may take the form of dilated cardiomyopathy or left ventricular systolic dysfunction(31, 32) (33). Patients with MDC1A have also been reported with cardiac abnormalities with one study suggesting subclinical cardiac involvement in 3-35% of cases (34, 35).

Eye involvement is a feature of some CMDs. This is particularly evident in the dystroglycanopathies where structural abnormalities such as anterior chamber defects, retinal abnormalities, cataracts and severe myopia are typical, particularly among the more severely affected individuals. In MDC1A, external ophthalmoplegia is also a recognised feature and whilst visual function is usually normal, visual evoked responses are usually abnormal (36).

Structural and functional brain involvement is common in the CMDs. This may exhibit as white matter changes on MRI brain scan associated with normal intelligence, as often seen in MDC1A, through to dramatic structural changes including agyria inevitably associated with profound mental retardation, as seen in WWS. Other features include seizures, often seen in MDC1A and learning difficulties in the absence of abnormal features on MRI brain scan. Conversely, intelligence and MRI brain imaging may be normal, as in the case of UCMD and BM.

1.5 INVESTIGATION AND DIAGNOSIS OF CMD

The investigation of CMD requires a detailed patient history and clinical assessment combined with directed pathological, biochemical and genetic testing and in many cases is supported by imaging techniques.

PATIENT HISTORY AND EXAMINATION

The baseline assessment for patients with CMD includes a detailed medical and family history and clinical examination. Features particularly relevant for CMD include age of onset, pattern of weakness and contractures, muscle wasting and hypertrophy, eye and brain involvement, presence of skin changes, feeding difficulties and respiratory and cardiac abnormality. Family information is important, in particular a history of consanguinity given the autosomal recessive nature of the majority of CMDs.

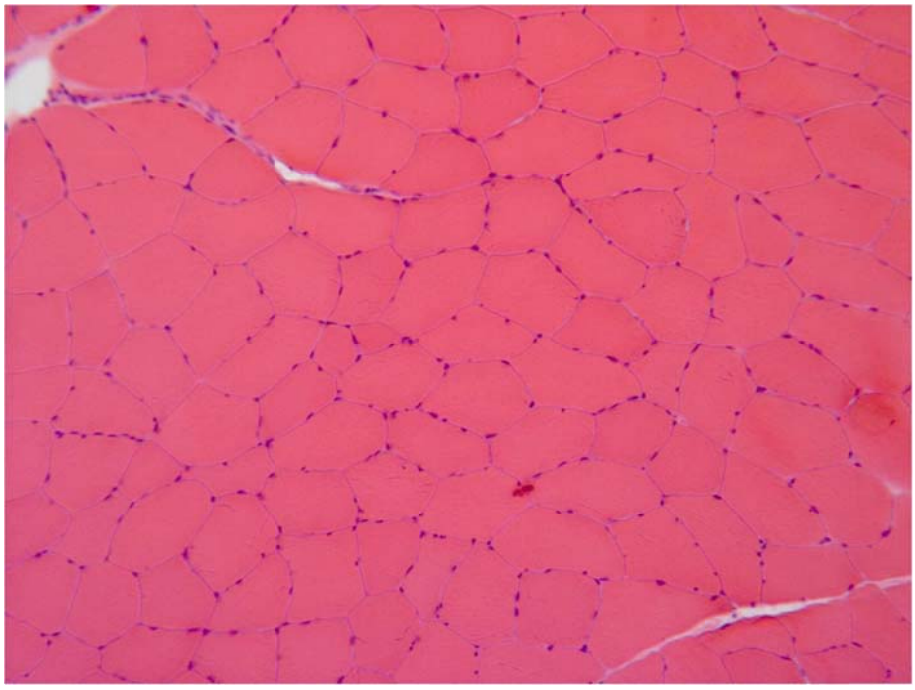
SKELETAL MUSCLE BIOPSY

Skeletal muscle biopsy forms a key role in the diagnostic assessment of patients with CMD. Skeletal muscle samples may be obtained by open or by closed (needle) biopsy procedure. In theory any muscle can be sampled but in practice it is usually the quadriceps muscle and historically in our department needle biopsy has been the most commonly used method of obtaining samples.

Biopsy samples can be assessed using a variety of immunohistochemical techniques depending on the diagnosis in question(4). Few findings are pathognomonic of a condition and it is therefore imperative that muscle biopsy findings are interpreted in conjunction with clinical patient review. The morphology of frozen transverse sections of skeletal muscle is routinely assessed using haematoxylin and eosin (H&E) staining. This allows assessment of, among others, the general structure of the muscle, the fibre size, presence of adipose tissue, inflammation, nerves and blood vessels. Other routine histochemical analysis allows the identification of enzyme abnormalities, substrate excess and other structural changes. Immunochemical labelling for specific proteins is increasingly used in the investigation of CMD and may provide a definitive diagnosis in some cases (for instance the absence of laminin α2 seen in MDC1A).

Although termed congenital muscular dystrophies, a dystrophic appearance on skeletal muscle biopsy is not universal and not a prerequisite for diagnosis of CMD. There are numerous reports of patients with CMD with minimal change or myopathic features on muscle biopsy. The cardinal features of dystrophy on skeletal muscle biopsy are fibrosis and necrosis. If necrosis is not seen then features of regeneration should be sought (CA Sewry, personal communication). A number of other features are frequently observed and may include variability in muscle fibre size, perimysial and endomysial proliferation, phagocytosis and increase in adipose tissue. Changes may be marked or mild and are not a reliable indicator of disease severity. In addition, muscle involvement may be selective and change with disease progression, hence biopsy findings are dependent on the individual muscle sampled as well as the age of the patient at time of biopsy (Figure 3)(4).

A



B

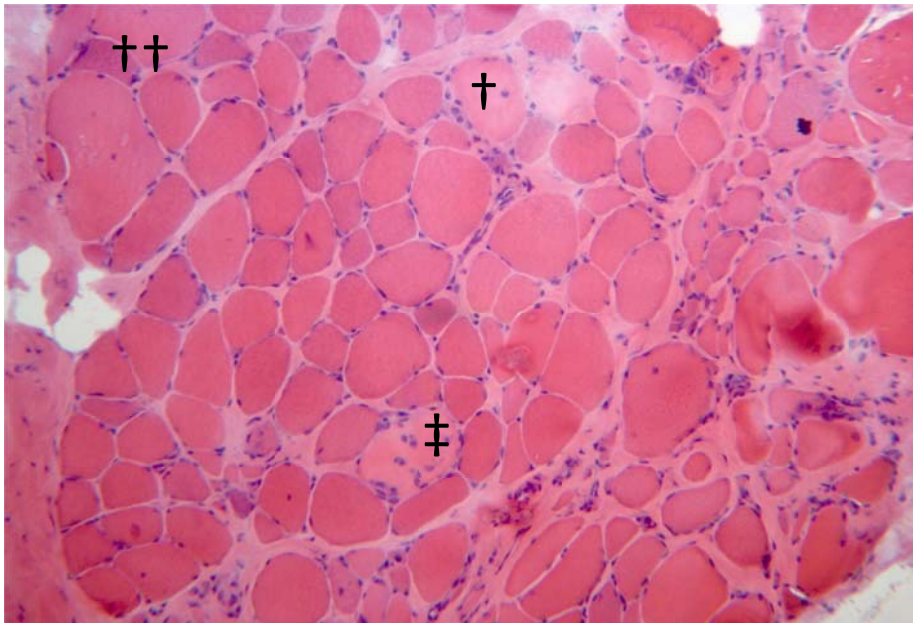


Figure 3. Normal and Dystrophic skeletal muscle.

Haematoxylin and eosin staining of normal muscle (A) and dystrophic muscle (B). Normal myofibres are approximately equal in diameter with nuclei located around the periphery (A). Dystrophic muscle is characterised by an accumulation of fat and connective tissue between fibres, increased variability in fibre size, a rounded morphology of cells, the presence of central nuclei (†), pale necrotic fibres (‡) and the presence of regenerating fibres (basophilic fibre††) (37). Images from Dr Lucy Feng, Dubowitz Neuromuscular Centre, Institute of Child Health, UCL.

SKIN BIOPSY

In some patients a muscle sample may not be available and in such cases pathological analysis of skin biopsy may help. In MDC1A, for example, laminin α2 may be reduced or absent in the epidermal-dermal junction. Secondary reduction can also occur however, so results should be interpreted with caution (4).

FIBROBLAST ANALYSIS

The use of fibroblasts cultured from skin biopsy is increasingly used in investigating CMD. They are of particular value in the investigation of Collagen VI related myopathies. Sometimes fibroblasts are used purely to extract RNA for protein expression studies. In other cases collagen VI expression may be analysed in cultured fibroblasts and although technically difficult has been shown to be a sensitive indicator of abnormal collagen VI expression (38, 39).

MOLECULAR GENETIC TESTING

The gold standard diagnostic test in the investigation of CMD is the identification of a pathogenic genetic mutation. All genetic testing has limitations and in the diagnostic setting screening of genomic DNA may miss deletions, duplications and inversions. Despite these general limitations, for some of the known CMD genes, genetic testing is straightforward. Screening of *SEPN1*, *FKRP* and *LAMA2* for example, is well established and the interpretation of results is usually uncomplicated. In other genes, genetic investigations are more difficult to interpret. In Collagen VI in particular, assessing which changes are pathogenic and which are polymorphisms is complicated. This is due to the size and complexity of the collagen VI genes, the variable clinical phenotype and mode of inheritance of the disorder. Many changes identified are currently classified as unknown variants. In such cases, alternative supportive evidence of pathogenicity is required and this may include RNA studies and family segregation studies in conjunction with consistent pathological and clinical investigations.

CREATINE KINASE

This is one of the most basic but most useful investigations in supporting a diagnosis of CMD. Serum Creatine Kinase (CK) is elevated in a number of CMDs and reflects muscle damage. The extent to which it is elevated may provide a clue to the diagnosis. For instance, in the dystroglycanopathies CK would be expected to be very elevated, sometimes up to 50X the upper limit of normal whilst in Collagen VI myopathies and RSMD1 the CK is usually only mildly elevated and may be normal.

MUSCLE IMAGING

On a basic level, the use of muscle ultrasound in the clinic setting may help by confirming the presence of increased echogenicity in muscles, indicative of muscle atrophy in patients with CMD. In recent years, our department and others have investigated and promoted the use of muscle magnetic resonance imaging (MRI) in the investigation of muscle disorders. The underlying principal is that abnormal increased signal intensity in muscle on T1 weighted images denotes fatty infiltration or atrophy. Different subtypes of CMD and congenital myopathy often have a specific pattern of muscle involvement (Figure 4). MRI Images taken through the transverse plain of the thigh and upper arm may reveal a characteristic pattern of abnormality and help direct molecular testing. Although not covered in detail within this thesis, myself and others have recently reported the sensitivity and specificity of muscle MRI changes found in 83 muscular dystrophy patients associated with rigid spine and also reported the muscle MRI appearance in FHL1 linked reducing body myopathy (40, 41).

BRAIN IMAGING

Several of the CMDs have associated brain abnormalities. These can have dramatic appearances on MRI brain scans and can be pathognomonic for a particular condition. Patients with MDC1A universally have diffuse white matter involvement seen after 6 months of life, often in conjunction with other abnormalities. Patients with dystroglycanopathies may have a variety of changes, the most striking of which is cobblestone lissencephaly. Some changes, in particular ventricular dilatation, may be apparent on foetal scans and in this regard can serve as a useful tool for prenatal assessment of a potentially affected baby (42).

ELECTROPHYSIOLOGY

In some variants of CMD, particularly MDC1A, abnormalities may be observed on electrophysiological testing. These patients have a motor demyelinating neuropathy and reduced nerve conduction velocity. In addition visual and somatosensory evoked responses are usually abnormal (36, 43).

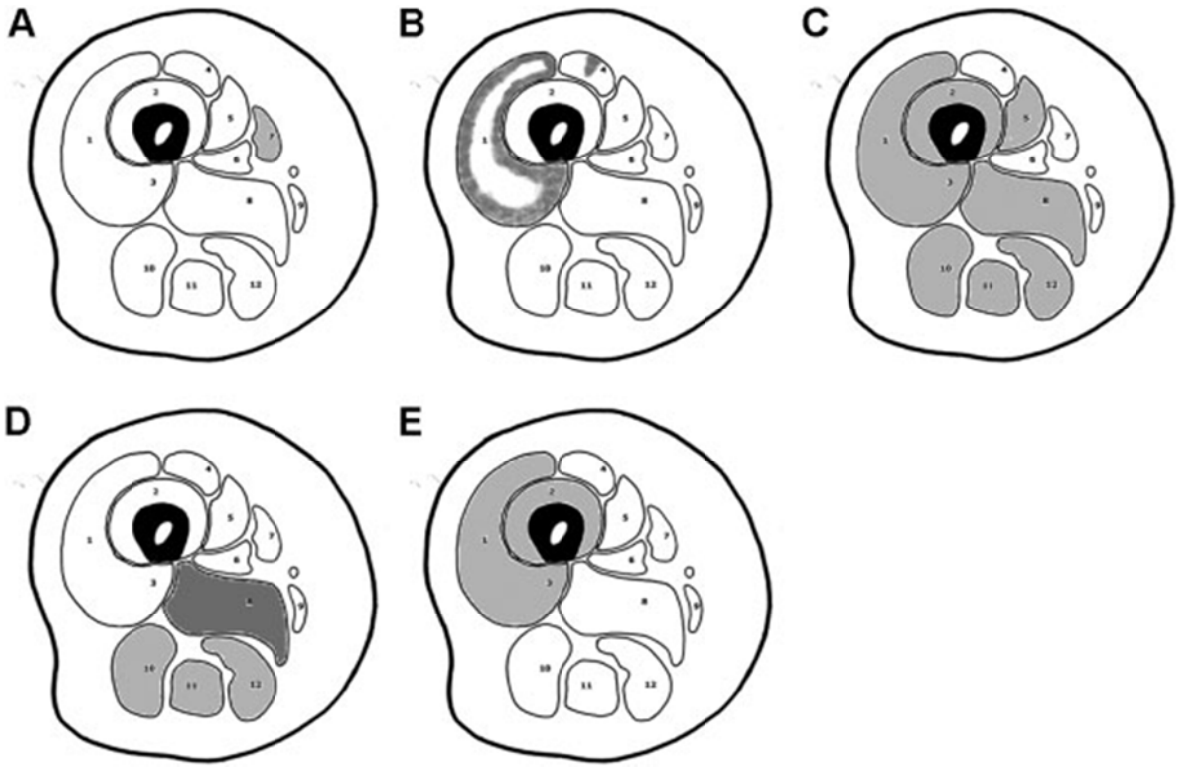


Figure 4. Diagrammatic representation of muscle involvement in CMD, as seen on MRI images of the thigh.

(A) Rigid Spine Muscular Dystrophy, (B) Bethlem Myopathy, (C) Ullrich Congenital Muscular Dystrophy, (D) Limb Girdle Muscular Dystrophy Type 2A and (E) Autosomal Dominant Emery-Dreifuss Muscular Dystrophy. Only the predominant muscle involvement is shown. 1, 3 = vastus lateralis; 2 = vastus intermedius; 4 = rectus femoris; 5 = vastus medialis; 6 = adductor longus; 7 = sartorius; 8 = adductor magnus; 9= gracilis; 10 = biceps femoris; 11 = semimembranosus; 12 = semitendinosus.

Figure adapted from Mercuri ***et al***, (40) reproduced with permission from John Wiley and Sons.

1.6 CONGENITAL MUSCULAR DYSTROPHY VARIANTS

In order to place my work in context, in 2006 when this project started, 16 CMD genes and 3 CMD loci had been reported (Table 1 and 2). There were also a number of CMD variants reported in the literature for which no gene or loci had been identified. Over the last 4 years, one new gene, Dolichyl-phosphate mannosyltransferase polypeptide 3 (DPM3), has been reported that is mutated in CDG1o, a condition with features overlapping CMD and a congenital disorder of glycosylation (CDG)(44). In addition, the 3p23 loci causing CMD with joint hyperlaxity (26) has been refined to reveal mutations in Integrin α9 (ITGA9) and the CMD associated with 4p16.3 (27) has been shown to not be a CMD at all, but a congenital myasthenia resulting from mutations in *DOK7* (Francesco Muntoni, personal communication). Mutations in Nesprin-1 are now known to be the cause of CMD with adducted thumbs, a phenotype initially reported by Voit *et al (25, 45)*. Mutations in the same gene also cause a form of autosomal recessive arthrogryposis with features overlapping CMD and congenital myopathy (46).

In 2006, CMD variants were broadly be grouped according to those that occur as a result of abnormalities in external membrane, basal lamina and extracellular matrix proteins (MDC1A, CMD due to integrin alpha-7 deficiency and collagen VI related myopathies) and dystroglycanopathies resulting from abnormal glycosylation of alpha dystroglycan (ADG). Other CMD phenotypes include RSMD1 resulting from mutations in *SEPN1*, a gene that encodes an endoplasmic reticulum protein of unknown function and EDMD2 resulting from severe mutations in the nuclear envelope protein Lamin A/C (12) (22). Each of these variants is detailed below, to provide a background to the work in this thesis.

1.6.1 Merosin Deficient Congenital Muscular Dystrophy Type 1A

Merosin (or laminin 2) deficient congenital muscular dystrophy type 1A (MDC1A, MIM #607855) was the earliest CMD pathologically defined due to a characteristic deficiency of merosin on skeletal muscle biopsy; this also became the first CMD variant for which the genetic defect, mutations in *Laminin α2 (LAMA2,* MIM #156225*)*, was found. Initially called classical or occidental CMD, it was previously thought to account for up to 50% of all cases of CMD. The availability of a straightforward pathological diagnosis and genetic testing, led to the rapid proliferation of manuscripts reporting the clinical features and relative frequency of this condition in several populations (47) (10) (9).

Laminin structure and function

Laminins are situated in the cell basement membrane and act as barriers to cell penetration and filtration, enabling the tissue to compartmentalise. Laminins are heterotrimers, formed from combinations of different α, β and γ chains. At least 15 different combinations are known to exist. The predominant form of laminin found in skeletal muscle basal lamina is laminin-2 (merosin) which is composed of α2, β1 and γ1 chains. The chains bind together via their coiled domains and through multiple interactions with other proteins play an essential role in maintaining basement membrane integrity during development and adult life. Merosin is necessary for myogenesis as well as for the survival and stability of myotubes in vitro. Muscle fibre degeneration and apoptosis occurs in the very early stages of MDC1A(48).

The laminin α2 chain consists of 6 domains: I and II are part of the long arm, IIIa, IIIb and V contain cysteine rich EGF like repeats, IVa, IVb and VI are predicted to form globular structures. The G domain, C-terminal region comprises internal homologous repeats and contains the interaction sites for membrane receptors that are, in muscle, α dystroglycan and integrin α7, an interaction necessary for the polymerisation of laminin (49). In skeletal muscle, laminin α2 is found around the muscle fibre, the Schwann cell basal lamina and at the neuromuscular and myotendinous junction (50) Although not expressed in the skeletal muscle blood vessels it is expressed in the basement membrane of brain blood vessels, epithelial cells lining the choroid plexus, oligodendrocytes tracts and glia limitans (51). The α2 chain is involved in cellular attachment, neurite growth and the migration of Schwann cells.

Clinical Features

The variant MDC1A is due to mutations in the *LAMA2* gene at 6q22, which encodes the laminin-α2 chain which forms part of merosin (Laminin-2) and also laminin-4. Most mutations result in complete absence of laminin α2 protein, with a small minority causing a partial deficiency. MDC1A with complete absence of merosin is a severe CMD, with CK levels typically exceeding 1000 U/l. It presents in the neonatal period or first few months of life with profound proximal and axial weakness, hypotonia and delayed motor milestones. Affected individuals rarely acquire independent ambulation and although most are able to sit unsupported, mobility is further compromised by the development of contractures and scoliosis. Most patients go on to need ventilatory support and enteral feeding and respiratory insufficiency, if left untreated, frequently leads to death in the first decade of life. Other features reported in MDC1A include partial external ophthalmoplegia and occasional cardiac abnormalities in the form of cardiomyopathy, hypokinesis or subclinical cardiac involvement.

Intelligence is usually normal in MDC1A although some have mental retardation and seizures. MRI brain imaging invariably reveals diffuse white matter changes after 6 months of life (9, 52, 53), thought to be dysmyelination, a change not seen in the *dy* (laminin deficient) mice. Structural brain abnormalities may include hypoplasia of the cerebellum (up to 1/3) and occasionally neuronal migration abnormalities, usually of the occipital lobes (54).

Laminin α2 is expressed in the Schwann cell basal lamina and both MDC1A patients and laminin α2 deficient mice have reduced nerve conduction velocity. In *dy* mice this may be due to disrupted Schwann cell basal lamina and defective myelination, but in humans Schwann cell basement membranes appear to be well preserved(55).

Genotype Phenotype Correlations

Mutations in *LAMA2* have been reported throughout the gene. Complete absence of Laminin α2 causes a severe predictable phenotype and may be caused by substitutions, deletions, or insertions in *LAMA2*. Some mutations have been seen repeatedly such as the 703X (2098delAC) and the C967X. The phenotypic spectrum seen in patients with partial deficiency is wide. Some patients display a milder clinical picture, whilst others, such as those with missense mutations in the conserved cysteine residues involved in trimer assembly, result in partial deficiency with a severe

phenotype. Mutations involving the G domain often result in a severe MDC1A phenotype because they interfere with the interactions with ADG and/or integrin α7/ β1D (48, 56-59).

Pathology and Diagnosis

Diagnosis in MDC1A is usually made by a combination of clinical features, muscle biopsy examination and genetic studies (either sequencing or linkage studies). Muscle biopsies in MDC1A patients show a variable degree of fibre necrosis and regeneration, increased connective tissue and inflammatory infiltrate. Immunocytochemically, laminin α2 chain is deficient with over expression of a4 and a5 chains in the basal lamina surrounding myofibres. In analysing the biopsies for MDC1A, antibodies must be used that recognise both the 80kDa C terminal and 300kDa N terminal fragments of laminin α2 or a reduction in labelling can be missed (60). Western blotting can also be used to detect laminin α2 defects. To complicate matters, in Laminin α2 deficiency, ADG and integrin a7b1 (both functional laminin receptors) show secondary reduction in labelling (61). When laminin α2 is absent from muscle basal lamina, it is also absent from the epidermal-dermal junction. Quantitatively, lamininα2 expression may be different in the muscle and skin from the same patient. Although expression of laminin α2 levels in the skin may be useful, a secondary reduction in the skin may also occur in conditions such as dystroglycanopathies secondary to *FKRP* mutations. The main differential in cases with reduction in Laminin α2 are the dystroglycanopathies where secondary reduction can occur with brain changes on MRI scan that are often similar to those seen in MDC1A.

Prenatal testing by CVS is also available in families with complete merosin deficiency due to abnormal laminin α2 expression in the trophoblast of affected individuals (62, 63). It is important in such cases to verify complete absence or dramatic reduction of merosin in the proband to avoid ambiguity in interpreting the results.

1.6.2 Collagen VI Related Disorders

The collagen VI related disorders, Ullrich Congenital Muscular Dystrophy (UCMD, MIM #254090) and its milder allelic variant Bethlem Myopathy (BM, MIM #158810), represent another significant proportion of CMD cases and are caused by mutations in *Collagen 6A1* (MIM #120220)*, 6A2* (MIM #120240) and *6A3* (MIM #120250). UCMD was first described in 1930 but the genetic defect was not reported until 2001 (64, 65).

Collagen VI Structure and Function

Collagen VI is a ubiquitously expressed extracellular matrix protein that forms a link between the cytoskeleton and extracellular matrix in skeletal muscle (66-68). Collagen VI is composed of 3 chains; α1 and α2 encoded by *COL6A1* and *COL6A2* on chromosome 21q2 and α3 encoded by the larger *COL6A3* on chromosome 2q3. All three chains contain a short triple helical domain with repeating Gly-Xaa-Yaa sequences flanked by globular domains (69, 70).

The collagen chains undergo a complex assembly process, initially forming triple helical monomers which then assemble into antiparallel dimers and then tetramers stabilised by disulphide bonds. These tetramers are secreted and associate extracellularly via N terminal globular domains to form a microfibrillar network in the reticular layer of basement membranes, collagen VI is also found in the endomysium and perimysium (71, 72). The myofibrillar network encircles interstitial collagen fibres and is in contact with the basement membrane surrounding muscle fibres (73-75). Co localisation experiments have suggested that one of the main functions of collagen VI is a structural role, anchoring the basement membrane to the underlying connective tissue by way of association with collagen IV (73). Collagen VI has also been shown to interact with cells directly via binding to transmembrane proteins such as integrins and NG2 proteoglycan and indirectly via basal lamina components including collagen IV, perlecan and decorin (76-79). In addition, *in vitro* experiments have shown a possible role for collagen VI in cell signalling (80-82).

Clinical Features

UCMD was originally described as an autosomal recessive condition, although autosomal dominant cases are increasingly recognised. It is characterised by neonatal onset proximal muscle weakness and contractures with distal joint laxityPresenting features may also include hypotonia, congenital dislocation of the hip, torticollis and

kyphosis. Motor achievement is variable; some patients achieve independent ambulation but this is usually lost and functional motor ability is further compromised by the development of contractures, scoliosis and spinal rigidity. Respiratory insufficiency requiring ventilatory support is almost invariable by the second decade of life but cardiac abnormality is not a feature. Intelligence is normal. The development of characteristic skin changes including hyperkeratosis and abnormal scarring may facilitate a diagnosis. CK is usually normal or mildly increased (39, 64, 83-85). Muscle MRI changes are characteristic and include diffuse involvement of the thigh muscles with relative sparing of the anteromedial muscles (Figure 4) (86).

Bethlem myopathy, usually an autosomal dominant entity, is a much milder disorder. It is reported as a slowly progressive proximal myopathy with the development of contractures particularly of the wrist, elbows, ankles and long finger flexors. Hypermobility of the distal interphalangeal joints is also a feature. In some cases BM may only be recognised in a parent after the diagnosis of the condition in a more severely affected child. Although the phenotype is milder than that of UCMD, patients may present in first years of life, usually with hypotonia, torticollis or congenital dislocation of the hip. Cardiac involvement is not a feature but patients may rarely develop respiratory insufficiency in later life (87, 88). Skin changes may be similar to those seen in UCMD. CK is normal or only mildly elevated (85).

Muscle MRI is often helpful in differentiating BM from other muscular dystrophies. Although changes are not always seen, especially in milder cases, a classical BM scan would show concentric muscle atrophy with peripheral involvement most evident in the vasti and gastrocnemii. There is often a characteristic ‘central shadow’ in the rectus which facilitates a diagnosis (Figure 4)(86, 89).

Although many patients with mutations in collagen VI are easy to classify as BM or UCMD, it is increasingly apparent that patients exist whose symptoms fall at the severe end of BM or the milder end of UCMD and display an ‘intermediate’ phenotype. This in turn led to the concept that BM and UCMD, rather than being separate disease entities, were in fact part of a spectrum of collagen VI related disorders (2).

Genotype Phenotype correlation

Mutations in all three genes cause BM and UCMD. The complex structure of collagen VI, the numerous polymorphisms in the 3 genes and the high number of splice site

mutations make mutation detection a lengthy task (90). RNA sequencing has become a commonly adapted strategy to overcome some of these obstacles.

A frequent group of mutations seen in BM are those that disrupt the Gly-Xaa-Yaa triple helical motif. It is thought that these mutations cause kinked collagen VI tetramers, reducing their ability to form microfibrils and resulting in BM by a dominant negative effect (91). Splice site mutations leading to skipping of exon 14 of *COL6A1* and subsequent deletion of 18 amino acids from the triple helical domain of the α1 chain are also frequently seen in BM. These mutations result in the production of shortened α1 chains that are unable to undergo subsequent assembly into dimers and tetramers and consequently cause a reduction in the detectable level of collagen VI in the extracellular matrix (92-94). Numerous other missense and splice site mutations have been reported although in many cases the pathogenicity of the variants has not been definitively established. (85)

In UCMD, mutations are often loss of function. These are frequently caused by the introduction of premature stop codons, by a variety of pathogenic mechanisms, and subsequent nonsense mediated RNA decay leads to loss of the mutant COLA chain (65, 85). Both autosomal recessive and dominant mutations cause UCMD with approximately equal frequency. A large heterozygous mutation in *COL6A1* has been shown to result in the secretion of abnormal tetramers that disrupt microfibrillar assembly by a dominant negative effect (94). Large inframe deletions and a number of missense mutations have also been reported in UCMD, the mechanism by which many of these mutations cause UCMD is not clear (85, 95).

As more mutations were discovered it became increasingly clear that a clean line could not be drawn between those mutations that cause UCMD and those that result in BM. Although single glycine substitutions in the triple helical domain usually result in BM, there are reports of UCMD patients with these as the only mutation. This supports the notion of a disease continuum between BM and UCMD (90).

Pathology and Diagnosis

Skeletal muscle biopsy in UCMD and BM may be dystrophic or myopathic. Immunohistochemical studies of collagen VI in BM and UCMD are variable and may show anything from a subtle reduction in the basal lamina to complete absence of extracellular matrix collagen. In BM, collagen VI immunohistochemistry in skeletal muscle biopsies is usually normal and therefore not of diagnostic use (84, 96, 97). In

UCMD however, complete or partial depletion of collagen VI may be observed and hence facilitate a diagnosis.

Collagen VI is also expressed in other connective tissue including skin. Immunostudies of collagen VI expression in skin may reveal a reduction although normal expression does not exclude a collagen VI defect. The analysis of fibroblasts derived from skin biopsies appears to be a more sensitive test of collagen 6 abnormality and has been used in diagnostic settings although is a complex technique and requires interpretation by experienced pathologists(38, 39, 71).

Immunolabelling of collagen VI in CVS samples has been successfully used in prenatal diagnosis of UCMD, but interpretation of collagen VI expression levels requires knowledge of the collagen VI status of the proband and should be linked to genetic data (98).

1.6.3 Rigid Spine Muscular Dystrophy

Mutations in *Selenoprotein N,1* (*SEPN1;* MIM #606210) give rise to 4 pathological phenotypes; rigid spine muscular dystrophy (RSMD1, MIM #602771), desmin-related myopathy with Mallory body-like inclusions (MB-DRM, MIM #602771) multiminicore disease (MmD, MIM #602771) and congenital fibre-type disproportion (CFTD MIM #255310). All are autosomal recessive conditions and although RSMD1 is typically the only one considered a CMD they show considerable phenotypic overlap (99-103).

SEPN1 Structure and Function

SEPN1 was the first human selenoprotein shown to be associated with a disease (102). It is a membrane bound glycoprotein found in the endoplasmic reticulum, with a high expression in foetal tissues but found at lower expression levels in a number of human tissues including skeletal muscle. The specific function of SEPN1 is unknown but it is thought to have a role in early development and in cell proliferation and regeneration (104). Recent work by Arbogast *et al.* has suggested an important role for selenoprotein N in protecting cells from oxidative stress damage(105).

Clinical Features

RSMD1 was first reported by Dubowitz in 1973 (106). It is characterized by early onset hypotonia and marked spinal rigidity as a result of contractures of the spinal extensors leading to loss of movement of the spine and the thoracic cage. Weakness is classically axial with head drop a finding in some. Facial and bulbar muscle weakness is frequently found and palatal weakness with a nasal sounding voice is common. Patients often have a thin habitus. Respiratory involvement is prominent and disproportionate to the muscle weakness which is often well preserved with many patients remaining ambulant into adult life. Assisted nocturnal ventilation is usually required in childhood or early adolescence. Structural or functional brain involvement is not a feature of RSMD1. CK is usually normal or only mildly elevated (102, 103, 107, 108).

Muscle MRI findings are characteristic with prominent involvement of the Sartorius and to a lesser degree adductors and biceps femoris (Figure 4) (40).

Genotype Phenotype Correlation

The diagnostic gold standard in RSMD1 is the finding of mutations in *SEPN1*. Mutations have been found throughout the coding region with no precise genotype phenotype relationship reported, although familial phenotype seems to be consistent. In addition no conclusive correlation has been reported between the muscle pathology and phenotype (99-103).

Pathology and Diagnosis

The pathological changes in skeletal muscle biopsy of RSMD1 patients are not diagnostic and as illustrated above, findings may be variable. Muscle histology may show non-specific myopathic features, multiminicores or congenital fibre type disproportion (108).

1.6.4 Autosomal Dominant Emery-Dreifuss Muscular Dystrophy

Autosomal Dominant Emery-Dreifuss Muscular Dystrophy (EDMD2, MIM #181350) is one of a number of disorders caused by mutations in *Lamin A/C* (*LMNA*, MIM #150330)(109). EDMD2 is not usually viewed as a congenital muscular dystrophy although early onset cases are documented.

In addition to EDMD2, *LMNA* mutations are responsible for, among others, Autosomal Recessive Emery Dreifuss (MIM #604929), Autosomal Dominant Dilated Cardiomyopathy (MIM #115200), Autosomal Dominant Limb Girdle Muscular Dystrophy type 1B (MIM #159001) and Autosomal Recessive Charcot Marie Tooth Disease (MIM #605588).

Lamin A/C Structure and Function

Three types of lamins, A, B and C, have been described in mammalian cells. Lamin A and C are derived from alternative splicing of the *LMNA* gene. *LMNB1* encodes lamin B (110). Lamins are intermediate filaments, structural proteins that are components of the nuclear lamina. Through their polymerisation and association with other proteins including emerin and nesprin, they form a network that determines nuclear shape and size and also make the nuclear envelope more resistant to mechanical stress (111). Many of the proteins associated with Lamin A are dependent on it for correct organisation (15) (112).

Clinical Features

EDMD2 classically presents with scapulohumeroperoneal atrophy and weakness, contractures of elbows and Achilles tendons and spinal stiffness. The major morbidity associated with the condition is the propensity to cardiac conduction defects that usually present before the third decade and may ultimately lead to complete heart block. Clinical variability is well documented with congenital presentations and slowly progressive adult forms reported. CK is normal or moderately elevated (2-20 x upper limit of normal).

Genotype Phenotype Correlation

The distribution and type of *LMNA* mutations found in EDMD2 is variable with a large proportion of *de novo* events. Mutations are found throughout the gene and many are

missense leading to amino acid substitution and a deleterious effect on protein folding and consequently the stability of the nuclear membrane. Nonsense mutations resulting in haploinsufficiency have only been reported in patients with striated muscle disease suggesting that these 2 mechanisms are the predominant cause of the *LMNA* associated skeletal muscle phenotypes (113). Significant clinical heterogeneity may exist even between patients with the same mutation. The weight of evidence suggests that disease modifying factors may be important in the pathogenesis of this group of conditions (109, 112-116). The mechanisms underlying this variability are unclear although it has been suggested that the more severe phenotypes are the result of dominant negative or gain of function mutations whilst the milder patients have loss of function mutations (117). Other work has highlighted the clinical variability found in patients with mutations in the same codon on exon 11 (118).

Pathology and Diagnosis

Skeletal muscle biopsy is not diagnostic and shows non-specific myopathic or dystrophic changes. A predominance of type 1 fibres may be present and structural changes such as cores may also be seen. Electronic microscopy may reveal specific alterations in nuclear architecture including aggregation of chromatin. Immunostaining for lamins in EDMD2 muscle biopsies, show no detectable difference from normal controls, as a result of the normal allele producing a normal product (119-121).

1.6.5 Congenital Muscular Dystrophy due to Integrin Alpha 7 Deficiency

Congenital muscular dystrophy due to integrin alpha-7 deficiency (MIM #613204) is a very rare form of congenital muscular dystrophy, with only 3 patients identified worldwide. It is due to mutations in *integrin alpha 7* (*ITGA7*, MIM #600536).

Integrins are transmembrane glycoproteins comprised of an α and β chain. Integrin α7β1 is a laminin α2 receptor in skeletal myotubes and mature myofibres. The 3 patients reported had motor delay with one patient also having mental retardation without brain MRI changes. CK was mildly elevated. Immunostaining of the skeletal muscle biopsy showed myopathic features with absent integrin α7 subunit and normal expression of laminin α2 (122, 123).

1.6.6 Congenital Muscular Dystrophy Type 1B

Congenital Muscular Dystrophy type 1B (MDC1B, MIM#604801) is linked to 1q42. Brockington *et al.* described 2 families with proximal girdle weakness, generalised muscle hypertrophy, contractures, spinal rigidity and early, severe respiratory failure. CK was very elevated and skeletal muscle biopsy was dystrophic with a secondary reduction in laminin α2 expression. Despite extensive investigation the gene responsible remains unknown (24, 28).

1.6.7 CMD with Joint Hyperlaxity

Tetreault *et al.* described a French-Canadian cohort of 14 patients with congenital muscular dystrophy and features overlapping those of the collagen 6 myopathies. In particular they had distal join laxity, contractures, hypotonia and a slowly progressive myopathy. Respiratory impairment was present but not as severe as that seen in UCMD and Bethlem Myopathy. Intelligence was usually normal and CK was normal or only mildly elevated. Collagen VI involvement was excluded by linkage and the disease was found to link to 3p23-21 presumably as a result of a founder mutation within that population. The defective gene is now known to be integrin α9 (29) (26).

1.6.8 Dystroglycanopathies

The dystroglycanopathies are discussed in more comprehensive detail than other CMDs as this group forms the basis for the majority of work in this thesis.

The dystroglycanopathies are a heterogeneous group of autosomal recessive disorders characterised by hypoglycosylation of alpha-dystroglycan (ADG) on skeletal muscle biopsy. They include CMD variants with structural changes affecting the brain and eyes (Fukuyama CMD (FCMD), Muscle-Eye-Brain disease (MEB), Walker-Warburg syndrome (WWS)), as well as relatively milder forms, characterised by subtle or absent brain involvement and ranging in severity from CMD (MDC1C, MDC1D) to later onset limb girdle muscular dystrophy (LGMD) forms (LGMD2I, LGMD2K, LGMD2L, LGMD2M) (2, 44, 124-126). By 2006, mutations in 6 genes (*Protein-O-mannosyl transferase 1 (POMT1*), *Protein-O-mannosyl transferase 2 (POMT2*), *Protein-O-mannose 1,2-N-acetylglucosaminyltransferase 1 (POMGNT1*), *Fukutin (FKTN*), *Fukutin-related protein (FKRP*) and *Like-Glycosyltransferase (LARGE*)) had been identified as giving rise to dystroglycanopathy phenotypes (127-132). Mutations in a further gene, *Dolichyl-phosphate mannosyltransferase polypeptide 3* (DPM3; MIM #605951) were reported in 2009 and give rise to condition with features overlapping those of the dystroglycanopathies and congenital disorders of glycosylation (44).

Structure and Function of Alpha Dystroglycan

During the 1990's a number of papers reported the importance of the dystrophin-associated-glycoprotein complex (DGC) in the pathogenesis of muscular dystrophies (133-137). The DGC is present along the sarcolemma of skeletal muscle fibres and contains a number of cytoplasmic, transmembrane and extracellular matrix proteins. Components of the DGC include: the cytoplasmic proteins dystrophin and syntrophins (α and β), the transmembrane protein sarcospan, the glycoproteins dystroglycan (α and β) and sarcoglycans (α, β, γ, and δ) (Figure 5) (138).

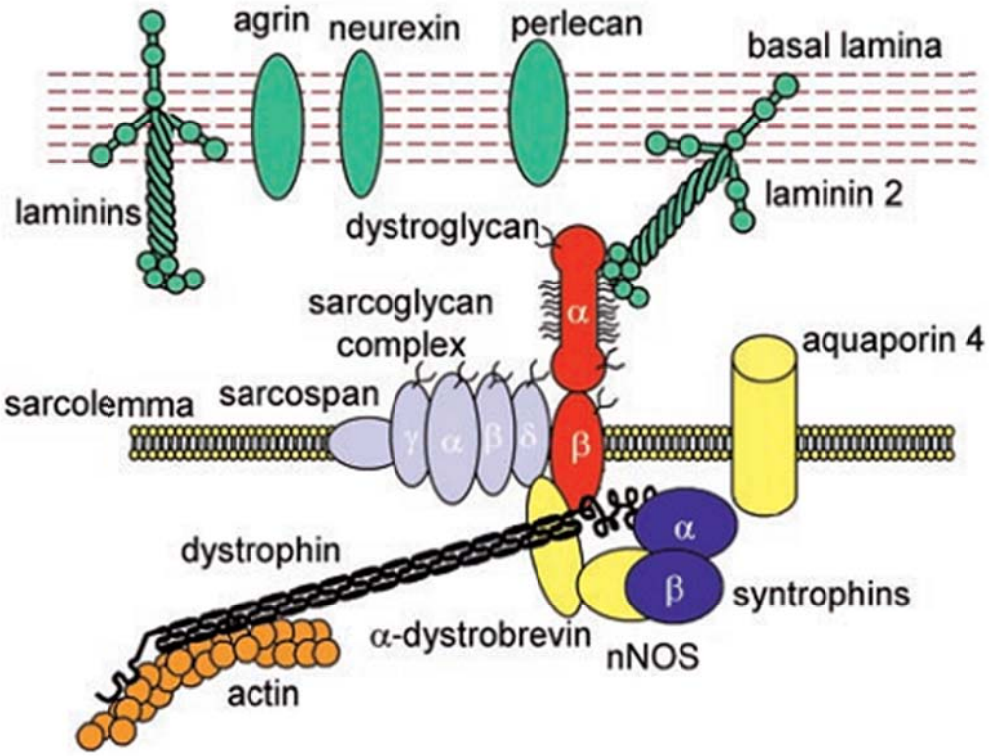


Figure 5. The skeletal muscle dystrophin-glycoprotein complex.

The components of the core dystrophin-glycoprotein complex include the cytoplasmic proteins dystrophin and syntrophins (α and β), the transmembrane protein sarcospan, the glycoproteins dystroglycan (α and β) and sarcoglycans (α, β, γ, and δ). The ligands for dystroglycan are shown in green.

This research was originally published in The Journal of Biological Chemistry. Michele DE and Campbell KP, Dystrophin-Glycoprotein Complex: Post-translational Processing and Dystroglycan Function. 2003; 278;15457-15460. © the American Society for Biochemistry and Molecular Biology. Reproduced with permission(14).

Central to the DGC are α and β dystroglycan (ADG and BDG), formed by posttranslational cleavage of the dystroglycan peptide. ADG is non-covalently linked to sarcolemmal spanning BDG that binds via its cytoplasmic domain to components of the cell cytoskeleton, hence forming a link between the ECM and cytoskeleton (139). The main function of the DGC in skeletal muscle is to confer structural stability to the sarcolemma during cyclical contraction and relaxation, acting as a 'shock absorber' protecting skeletal muscle from damage (140).

The gene encoding dystroglycan, *DAG1*, was initially cloned in 1992 and subsequently mapped to 3p21 in 1993 (141, 142). The primary structure of dystroglycan is strongly conserved across mammalian species and displays a high degree of homology throughout lower invertebrates. No mutations in *DAG1* have been identified in any human disorder although post-translational processing defects of dystroglycan, in particular abnormal glycosylation, have been associated with a number of pathological conditions. Dystroglycan has a wide tissue distribution and outside skeletal muscle is expressed in the central and peripheral nervous system, in epithelia and endothelia (143). Dystroglycan has diverse roles and in addition to its role in the DGC has been implicated in cell signalling (144, 145), structure and function of the central nervous system (146), myelination and nodal architecture of peripheral nerves (147), epithelial morphogenesis (148), synaptogenesis (149) (150) and cell adhesion (151).

In the DGC, transmembrane BDG binds intracellularly via its carboxy terminal residues to dystrophin via the WW (Trp-Trp) domain and Ca^{2+} binding motifs (152) which in turn bind to the actin associated cytoskeleton. In tissues other than skeletal muscle, dystroglycan binds to various alternative splice forms of dystrophin or utrophin (153). A for BDG role in cell signalling is also implied given interactions with the signalling adapter molecule Grb2 and components of the ERK-MAP kinase cascade including MEK and ERK. The precise biological significance of these interactions is unknown (145) (154).

ADG is an extracellular glycoprotein that contains a central, highly *O*-glycosylated, mucin domain connecting the globular C- and N-terminal domains. ADG also contains 3 potential N-linked glycosylation sites (155, 156). The predicted molecular mass of the core protein is ~40 kDa, however the size of ADG detected by immunoblot varies from 120 kDa in brain to 156 kDa in skeletal muscle, largely due to extensive *O*-glycosylation (138). ADG binds many of its extracellular matrix partners through its

carbohydrate modifications. Known ECM ligands include laminin-2, agrin and perlecan in skeletal muscle and neurexin and pikachurin in the brain and retina respectively (157) (158) (141). ADG is also the cellular receptor for arenaviruses and *Mycobacterium leprae* (159, 160).

Dystroglycan is expressed in high levels in developing and foetal tissues, typically in cell types facing basement membranes. Disruption of *DAG1* in mice is embryonically lethal around day 5.5 due to perturbations in Reichert's membrane, a basement membrane that separates the embryo from the maternal circulation (161). It was subsequently shown that dystroglycan is required for the formation of the sub-endodermal basement membrane in embryoid bodies and is also required for the organisation of laminin-1 on the cell surface (162). However in the Large^{Myd} mouse, (in which *LARGE* is mutated causing ADG hypoglycosylation), the skeletal muscle basement membranes are largely intact despite large disruptions of basement membranes in the brain (163). It is probable that the role of dystroglycan in basement membrane formation is dependent on numerous factors (154). Mice with skeletal muscle specific loss of dystroglycan showed mild muscular dystrophy with skeletal muscle hypertrophy. This suggested that maintenance of regeneration capacity by satellite cells expressing dystroglycan was likely to result in a milder muscular phenotype than that observed in chimeric dystroglycan null mice, implying a role for dystroglycan in skeletal muscle repair (164, 165).

Dystroglycan also has an important function at the Neuromuscular Junction (NMJ). Most proteins of the DGC are expressed along the entirety of the sarcolemmal membrane. At the extrasynaptic regions of the myofibre a protein scaffold similar but not identical to the DGC exists, containing proteins that are only expressed at the NMJ. This includes uniquely synaptic forms of laminin, utrophin and dystrobrevin-1. The localisation of dystroglycan at the NMJ is dependent on its interaction with ankyrin (166). Synaptic forms of laminin and agrin interact with ADG, whilst BDG interacts intracellularly with utrophin which binds to actin filaments. BDG also binds to rapsyn, a postsynaptic protein involved in clustering of acetylcholine receptors implying a role in the formation of neuromuscular junctions (167). Further work suggests a role for dystroglycan in the stabilisation of post-synaptic acetylcholine receptor clusters (168).

In the retina, distinct isoforms of ADG are localised in apposition to the basal lamina in the inner limiting membrane and blood vessels as well as within the parenchyma of the retina along the Muller glia. It is thought that dystroglycan has a role in organising

synapses and basement membrane assembly in the retina (169). Recently it has emerged that pikachurin (a ligand of dystroglycan) plays a crucial role in the formation of the photoreceptor ribbon synapse and that posttranslational modification of ADG is necessary for correct pikachurin binding and localisation (170).

The role of ADG in the developing brain and in neuronal migration has been studied in animal models. In adult mice ADG is expressed in the neurones of cerebral cortex, hippocampus, olfactory bulb, basal ganglia, thalamus, hypothalamus, brainstem and cerebellum. During CNS development, ADG is expressed in the ventricular zone and in basement membranes, and participates in neuronal proliferation, in the constitution of the meningeal layer and in neuronal migration (171). This is covered in more detail in section 4.1.1.

Dystroglycan is also thought to have a role in branching morphogenesis of kidney, lung and salivary glands and is expressed in high levels on the basal side of most epithelial cells (172, 173).

DGC proteins are implicated in the pathogenesis of a number of muscular dystrophies including dystrophinopathies and sarcoglycanopathies. Involvement of basal lamina proteins in the pathogenesis of dystroglycanopathies was first postulated to be important in patients with FCMD. Molecular work established the reduced expression of laminin α2 chain of merosin in the basal lamina of skeletal muscle from these patients and subsequently an abnormality in the glia limitans (a basal lamina complex in the brain) of foetuses (174, 175).

In 2001, Hayashi et al demonstrated reduced immunoreactive levels of ADG in FCMD patients using a VIA-4 antibody, directed against glycosylated ADG. They also reported loss of the 156kDa band, representing glycosylated ADG in skeletal muscle, in FCMD patients. BDG expression was normal in both immunoreactive studies and immunoblot. They thought that the most likely explanation for their results was that ADG was selectively lost in FCMD, perhaps as a result of protein instability or altered glycosylation (176). Around the same time, Grewal *et al*. showed that the gene mutated in the myodystrophy mouse (Large^{Myd}) encoded *LARGE*, a glycosyltransferase (177). Due to the muscular dystrophy phenotype of the Large^{Myd} mouse, DGC proteins, in particular those that are glycosylated were identified as potential downstream targets of *LARGE*. They showed that whilst migration of sarcoglycans and BDG was normal in immunoblots of Large^{Myd} skeletal muscle, monoclonal antibodies to ADG (VIA-4) showed almost complete loss of binding. This led to the speculation that abnormal

glycosylation of ADG could be implicated in muscular dystrophy. Further work by the same group went on to refine the phenotype of the Large^{Myd} mouse, revealing ophthalmic and CNS defects including neuronal migration abnormalities, features reminiscent of those seen in MEB and FCMD (178). Complementary work by Michele *et al.* used polyclonal antibodies directed against ADG to demonstrate that in MEB and FCMD patients, ADG was in fact expressed at the muscle membrane but was hypoglycosylated, accounting for the reduced levels of ADG seen when using VIA-4 antibodies. Such hypoglycosylation would interfere with ADGs ability to interact with extracellular ligands providing an elegant explanation for the muscular dystrophy phenotype. Michele *et al.* expanded the phenotype of the Large^{Myd} mouse, demonstrating the similarity in brain abnormalities, including disruption of the basal lamina, between the mouse and the MEB/ FCMD patients studied. They concluded that FKTN, LARGE and POMGNT1 may participate in a similar pathway resulting in the glycosylation of ADG. This discovery provided a neat explanation for the phenotypic overlap reported in MEB, FCMD and other 'dystroglycanopathy' patients with mutations in different genes (163).

Glycosylation

Glycosylation is highly complex post translational process by which sugars are added to proteins in the endoplasmic reticulum and Golgi apparatus. Glycosylation affects protein stability, conformation and function which in turn affect crucial cellular processes such as molecular recognition, cell adhesion, growth and differentiation (179, 180). Glycosylation occurs in 2 main forms. *N*-glycans are linked to the target protein via an asparagine residue whilst *O*-glycans are linked via a serine or threonine. ADG is both *N*- and *O*- glycosylated, the glycosylation of ADG is tissue specific and the molecular mass is different in skeletal muscle (156kDa), cardiac muscle (140kDa) and brain (120kDa).

N- glycosylation is by far the most common form of glycosylation seen in mammalian cells. Disorders that affect *N*-glycosylation and are known as congenital disorders of glycosylation (CDGs).They can be subdivided into type I and II. Type I CDGs are caused by defects in the assembly of lipid linked oligosaccharides in the endoplasmic reticulum resulting in the production of incomplete glycan structures. Type II CDGs are caused by defects in the processing of protein bound sugar chains and include conditions with mutations in nucleotide sugar transporters, glycosyltransferases, and cytoplasmic proteins that traffic into and within the Golgi apparatus (181, 182).

O-glycans can be divided according to the sugar that *O*-links with the protein. ADG is an *O*-mannosylated protein, a rare finding in mammalian cells (183). The central *O*-glycan sequence in ADG is – -Sialyl-α2,3Galβ1,4GlcNAcβ1,2Manα-*O*-Ser/Thr-. The –Sialyl-α2,3Galβ1,4GlcNAcβ1 portion is necessary for binding of ADG to laminin (184). Several other glycans have been identified that are derivatives of the *O*-linked mannose structure including the Lewis X antigen, the CT carbohydrate antigen, restricted to the neuromuscular junction and the HNK-1 carbohydrate antigen (185-187).

The formation of the major *O*-linked mannose glycan on ADG involves the action of specific enzymes (glycosyltransferases) that add monosaccharides in a stepwise manner (12, 188). The genes implicated in the dystroglycanopathies are putative or proven enzymes involved in the *O*-mannosylation of ADG (2). As such the hypoglycosylation observed in this group of conditions is a secondary effect as a consequence of abnormalities in glycosyltransferases rather than a primary abnormality in ADG. POMT1 forms a complex with POMT2 (132) that catalyses the first step in the assembly of the *O*-mannosyl glycan, adding a mannose directly to the Ser/Thr residue. POMGNT1 is the second enzyme in the *O*-mannosylation process, adding an *N*- acetylglucosamine (GlcNAc) residue to the mannose group. Interruption of the *O*-mannosylation pathway provided a clear explanation for the pathogenicity of mutations in these three genes however the precise function of FKTN, FKRP and LARGE in facilitating the glycosylation of ADG has yet to be elucidated (Figure 6) (129, 132, 189-191).

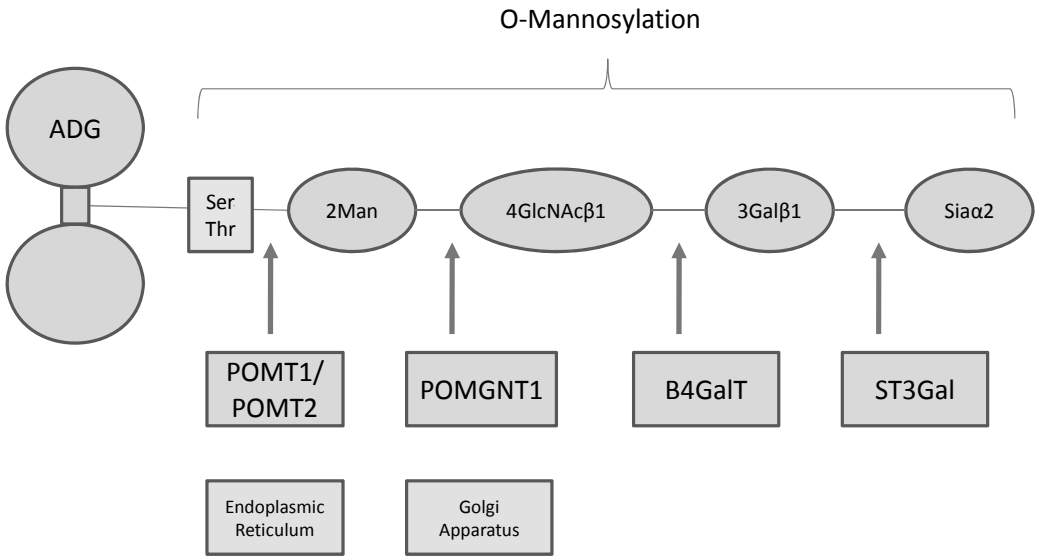


Figure 6. Diagrammatic representation of the O-mannose-linked glycan attached to ADG.

Sugar residues are added in sequential order to the ADG backbone by the catalytic activity of glycosyltransferases. POMT1 and POMT2 act in the endoplasmic reticulum and catalyse the first step, the addition of mannose (190). POMGNT1 catalyses the second step in the Golgi apparatus, the linkage of a N-acetylglucosamine residue to the mannose (129). The function of FKTN, FKRP and LARGE have yet to be fully characterised.

FKTN and *FKRP* show sequence similarity to glycosyltransferases(192, 193). *FKTN* is expressed in highest amounts in brain, heart, pancreas and skeletal muscle. Although FKRP is a putative glycosyltransferase, its precise subcellular localisation is controversial. Some studies have suggested that it localises to the rough endoplasmic reticulum with POMT1, whilst others report subcellular localisation of FKRP to the Golgi apparatus with mutations in *FKRP* leading to endoplasmic reticulum retention (193-196). In one study in mammalian cells FKRP was found to be present at the sarcolemma and associated with the DGC although the precise role of FKRP remains elusive(196).

The *LARGE* gene encodes the *N*-acetylglucosaminyltransferase-like protein (LARGE), a homologue of mammalian β3,1-N-acetylglucosaminyltransferase. The gene is ubiquitously expressed with the highest levels seen in heart, brain and skeletal muscle (197). The LARGE protein is localised to the Golgi and contains 2 putative catalytic domains, both of which are necessary for correct function(177, 198). The *N*-terminal domain of ADG interacts directly with LARGE and initiates functional glycosylation of ADG (199, 200). The precise action of LARGE is unknown. However, forced expression of LARGE induces hyperglycosylation of ADG in normal cells and also restores the hypoglycosylation of ADG in patients with dystroglycanopathies including FCMD, MEB, WWS and LGMD2I, resulting in increased laminin binding. Interestingly, hyperglycosylation of ADG can also be restored by overexpression of a LARGE homologue, LARGE2 (GYLTL1B). LARGE2, although sharing sequence similarity has a different expression pattern to LARGE, having virtually absent expression in skeletal muscle and heart(198). This interesting observation has opened up the possibility exploiting this phenomenon for therapeutic benefit in patients with dystroglycanopathies (158, 201). Work published this year by Yoshida-Moriguchi *et al*. has suggested that patients with MEB and FCMD as well as the Large^{Myd} mouse, have defects in postphosphoryl modification of a phosphorylated O-linked mannose in ADG. This modification is mediated by *LARGE* and is required for laminin binding, hence suggesting a mechanism by which forced overexpression of *LARGE* may circumvent defects in CMD cells (202).

Interestingly, aberrant glycosylation and post translational modification of ADG is implicated in the pathogenesis of other conditions including a number of cancers, probably as a result of disruption to the basal lamina and interaction with the extracellular matrix. Loss of ADG expression in prostate and breast cancer cells correlates with a higher degree of malignancy (158, 203, 204). Correct post translational processing is also necessary for ADG to act as a receptor for a bacteria

and viruses including lymphocytic choriomenigitis virus (LCMV) and Lassa Fever virus (LFV) (158, 159).

Dystroglycanopathy Phenotypes and Associated Genes

The phenotypes reported in patients with hypoglycosylation of ADG are very variable. The clinical classification of dystroglycanopathies has been clouded somewhat by the heterogeneity of this group. Initially, discreet phenotypes were described based on clinical findings and sometimes associated with a particular population; MEB was well described as a severe CMD with eye and brain abnormalities common in the Finnish community, FCMD was known as clinically similar to MEB but isolated to the Japanese population and WWS was recognisable by the devastating extent of the CNS phenotype. As the genetic basis for individual conditions emerged, they became associated with mutations in a particular gene; MEB with *POMGNT1*, FCMD with *FKTN* and WWS with *POMT1*.

As more dystroglycanopathy genes were discovered, additional phenotypes attributed to these new genes were reported, often with features similar to those described in other dystroglycanopathies. The first patient found to have a *LARGE* gene mutation for instance, was designated as having MDC1D, a condition with features of CMD and brain abnormalities in keeping with milder aspects of the previously reported conditions (131). As a consequence, a number of dystroglycanopathy phenotypes, particularly in the intermediate severity range, have overlapping features but have historically emerged as separate conditions. This classification problem was confounded further by the realisation that particular dystroglycanopathy genes may produce several different phenotypes, best illustrated by mutations in *FKRP* which may produce anything from WWS through to milder late onset LGMD without CNS involvement (205). As it currently stands, among the dystroglycanopathies, mutations in one gene may cause several phenotypes and some phenotypes may be caused by mutations in a number of different genes. In addition the diagnostic boundaries between some of the conditions, particularly in the intermediate spectrum are not clear. Despite this, several key phenotypes have been well described and these, together with the associated genes involved are summarised below.

Walker Warburg Syndrome (WWS, MIM #236670): This is a severe form of CMD with onset prenatally or at birth. Patients have structural brain abnormalities including complete agyria or severe ‘cobblestone’ lissencephaly with only rudimentary cortical

folding, marked hydrocephalus, severe cerebellar involvement and complete or partial absence of the corpus callosum. Eye abnormalities include congenital cataracts, microphthalmia and buphthalmos. Motor development is minimal or absent and death before 1 year of age is usual (206).

The first gene identified in patients with WWS was *Protein O-mannosyltransferase (POMT1)*, found to be mutated in 20% of a WWS patient cohort (130). In 2005, *Protein O-mannosyltransferase 2 (POMT2)*, a homologue of *POMT1*, was also found to be mutated in a number of patients with WWS. *POMT2* was an obvious dystroglycanopathy candidate gene as it shares 36% amino acid identity with *POMT1*. *POMT1* and *POMT2* are expressed in most human tissues and expression of both proteins is required for POMT enzymatic activity (190). *POMT2* was reported to be mutated in 3/41 patients with WWS. Mutations in *FKRP and FKTN* were subsequently identified in WWS patients. It has been estimated that mutations in *POMT1*, *POMT2*, *FKTN* and *FKRP* account for approximately 1/3 of patients with WWS (130), (132, 207, 208).

Muscle Eye Brain Disease (MEB, MIM #253280): MEB is a CMD with brain abnormality less pronounced than that seen with WWS, originally reported in the Finnish population. MRI brain findings include; cortical abnormalities including pachygyria and polymicrogyria, cerebellar abnormalities including hypoplasia, dysplasia and cysts, and brain stem abnormalities. Structural eye involvement is a feature and may include congenital glaucoma, progressive myopia, retinal atrophy and juvenile cataracts. Individuals may, rarely, acquire the ability to walk although this is delayed. Significant learning difficulties are expected although patients occasionally manage to learn a few spoken words (206).

The MEB locus 1p32-p34 was first identified in 1999 and was followed in 2001 by the report of *Protein O-linked mannose β1,2-N-acetlyglucosaminyltransferase 1 (POMGNT1*) mutations in Finnish and Turkish patients with MEB(129, 209). Cormand *et al*. subsequently analysed a large cohort of MEB and WWS patients and effectively excluded linkage to 1p32-p34 from all but 1 patient with diagnostic criteria compatible with WWS. Conversely all patients except 1 Swedish girl with features consistent with MEB linked to the locus. Mutations in *FKRP* and *POMT2* were subsequently found in MEB patients *(129, 210, 211)*.

Fukuyama Congenital Muscular Dystrophy (FCMD, MIM #253800): This is the second most frequent form of muscular dystrophy in Japan after Duchenne muscular

dystrophy, due to the presence of a *FKTN* founder mutation within this population. Reports of the disease outside of Japan are rare. FCMD was first described by Yukio Fukuyama in 1960 (212). Affected children have generalized muscle weakness with marked motor delay, most patients only achieve independent sitting or standing with support. Pseudohypertrophy of the tongue, calves, and quadriceps muscles is common. Brain involvement is reflected in structural brain malformations including cobblestone lissencephaly, more often described as pachygyria or polymicrogyria although all part of the same spectrum, dilated lateral ventricles, white matter abnormality seen on T2 weighted images indicative of dysmyelination, midbrain hypoplasia and cerebellar abnormalities including polymicrogyria and cysts (213). Functional brain involvement is found in the form of mental retardation and seizures. Respiratory failure in the mid-to-late teens is an invariable complication (127, 214).

Mutations in the *FKTN* gene were first reported in patients with FCMD in 1998 (127, 215). A common founder retrotransposal insertion was found in more than 80% of FCMD chromosomes.

Congenital Muscular Dystrophy type 1C (MDC1C, MIM #606612): A bioinformatics approach, searching for homologues of *FKTN* led to the discovery of mutations in *FKRP* in a group of patients with CMD and secondary reduction in laminin α2, designated MDC1C (128). They were subsequently found to have hypoglycosylation of ADG on muscle biopsy. Patients are characterised by pronounced muscle involvement with sparing of functional brain abnormality in most cases. Patients typically present in the first few months of life with hypotonia and weakness and do not acquire independent ambulation. Leg and tongue muscle hypertrophy are features and shoulder girdle wasting is often seen. Weakness is not particularly progressive but disability is compounded by the development of scoliosis and respiratory decline usually necessitating non-invasive ventilation in the second decade. Dilated cardiomyopathy is also a finding in some. *FKRP* mutations were subsequently reported in patients with MDC1C associated with mild mental retardation and cerebellar cysts (128, 211, 216).

Limb Girdle Muscular Dystrophy type 2I (LGMD2I, MIM #607155): This is a milder allelic condition to MDC1C, although the range of severity within this group is itself varied. Patients typically present in the second or third decade with gait abnormality resulting from limb girdle weakness. Facial and neck flexor weakness is also common and calf and tongue hypertrophy is often seen. Cardiac involvement is sometimes observed, usually left ventricular abnormality. At the more severe end of the spectrum,

patients may present in the first few years of life with delayed motor milestones in addition to the above features and follow a motor severity course comparable to Duchenne muscular dystrophy. Intelligence and brain MRI imaging is normal. CK typically ranges between 1000-4000 IU/l. A common C826A mutation in *FKRP* is frequently observed in this phenotypic group and patients who are homozygous for this mutation are usually at the milder end of the phenotypic spectrum (192).

Congenital Muscular Dystrophy type 1B (MDC1B, MIM #604801): This is a congenital muscular dystrophy reported in 2 consanguineous families characterised by weakness of the proximal girdle, facial muscles and sternomastoids with generalised muscle hypertrophy. The striking finding is of profound respiratory deficiency in the first decade of life resulting from diaphragmatic weakness and spinal rigidity. Conversely weakness elsewhere remains relatively non progressive. Intellect is normal with no evidence of central nervous system involvement and CK is characteristically raised (2000-7000 IU/l). This disease has been assigned to 1q42 although despite extensive searching the genetic defect has not been ascertained (24, 28).

Congenital Muscular Dystrophy type 1D (MDC1D, MIM #608840): This is the phenotype attributed to the first patient described with a *LARGE* mutation. *LARGE* was identified as a candidate gene for dystroglycanopathy because of the evocative phenotype in the Large^{Myd} mouse in which *LARGE* is mutated. This patient presented at 5 months of age with developmental delay and hypotonia. She progressed to independent walking at the age of 4 years and at the age of 17 years was able only to manage a few steps. She had moderate calf, arm and quadriceps hypertrophy and mild facial weakness. She had profound mental retardation with use of a few words without meaning. Brain MRI was abnormal with high signal intensity in the white matter, abnormal neuronal migration with pachygyria and a hypoplastic brainstem. Echocardiography was normal. Fundoscopy was normal although electroretinographic examination revealed reduced amplitude of the evoked response (131).

Limb Girdle Muscular Dystrophy type 2K (LGMD2K, MIM #609308): This phenotype describes patients, originally identified in the Turkish population, with a limb girdle muscular dystrophy presenting in the first decade of life, mental retardation and microcephaly without any structural brain abnormality detectable on MRI. This group of patients were subsequently found to harbour A200P mutations in *POMT1* (125, 217).

Congenital Disorder of Glycosylation type Io (CDG1O, MIM #612937): In 2009 Lefeber *et al.* reported a patient presenting with mild muscular dystrophy, dilated cardiomyopathy and stroke like episodes with no associated brain or eye involvement. IIH6 immunoreactivity was reduced on skeletal muscle biopsy, consistent with a dystroglycanopathy, but transferrin isoelectric focusing studies in blood also revealed an abnormal transferrin profile. This was in keeping with a CDG type I pattern consistent with a disorder in N-glycosylation and an ER defect. A reduction in Dol-P-Man synthase activity was found in the patients fibroblasts. Mutation analysis subsequently revealed a homozygous mutation in *DPM3*, one of 3 subunits in the Dol-P-Man synthase complex, required for anchoring the catalytic subunit to the ER. The Dol-P-Man synthase complex is required for *N*-glycosylation, *C*-mannosylation, *O*-mannosylation and GPI-anchor formation and a mutation would be expected to affect all 4 processes. It is speculated that *O*-mannosylation is more sensitive to the reduced binding capacity of DPM3 resulting from the reported mutation and consequently produces a phenotype that overlaps with that seen in dystroglycanopathy (44).

Prior to this report, CDGs and dystroglycanopathies, although both disorders of glycosylation, were regarded as two separate disease entities (44). This report reveals for the first time a patient with clinical features consistent with a dystroglycanopathy, supported by reduced ADG glycosylation in skeletal muscle, but with a biochemical profile in keeping with a CDG.

Genotype Phenotype Correlation

Initially a genotype-phenotype correlation was reported between the individual dystroglycanopathy genes and various phenotypes. However, subsequent work has shown that each of the genes is responsible for a wide range of clinically overlapping conditions as illustrated by the phenotypic descriptions above. Despite this, certain genes are classically associated with particular conditions; for instance, mutations in *FKTN* give rise to Fukuyama CMD in the Japanese population, almost invariably as a result of founder mutations. This is expanded further in section 3.1.

Pathology and Diagnosis

Skeletal muscle biopsy in patients with dystroglycanopathy is usually dystrophic in appearance with a large proportion of fibres expressing neonatal myosin. A reduction in ADG on immunoblot and immunohistochemistry is apparent with normal expression of BDG (12). The commonly used monoclonal antibodies (IIH6 and VIA-4) are directed

against the glycosylated epitope of ADG, and show variable reduction in labelling that does not consistently correlate with disease severity (12, 218). Other features include a reduction in labelling of laminin α2, although this is never absent as often seen in MDC1A (Figure 7)(128).

In order to investigate the specific involvement of POMGNT1, POMT1 and POMT2, enzymatic activity can be studied in muscle biopsy samples, fibroblasts or EBV-transformed lymphoblasts. These enzymatic assays are not routinely used in our department for diagnostic studies, but have been used to investigate cases where pathogenicity of a mutation is disputed (190, 219, 220).

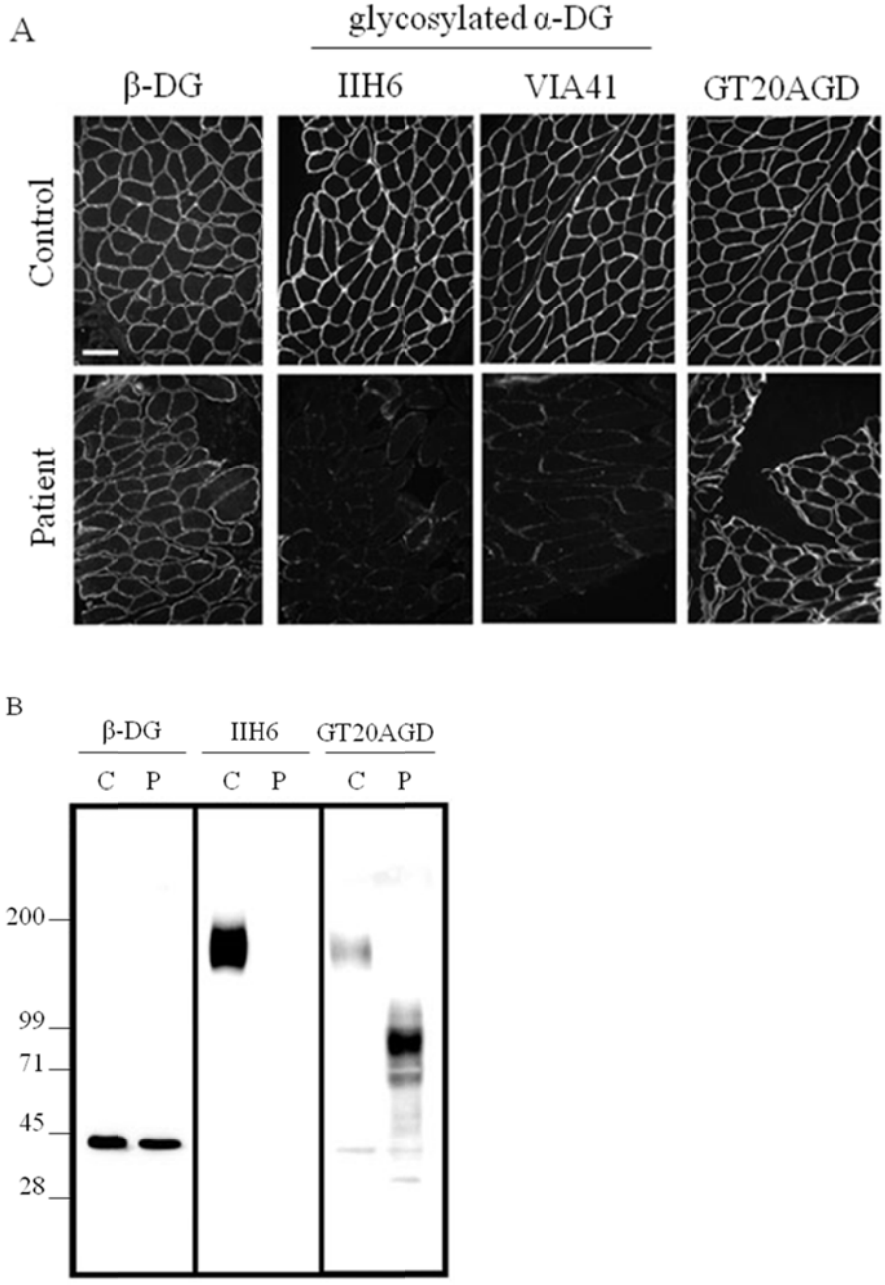


Figure 7. Hypoglycosylation of α-dystroglycan as detected on skeletal muscle biopsy.

Diagnostic features of dystroglycanopathy skeletal muscle using antibodies against β-dystroglycan; β-DG, carbohydrate moieties present on α-dystroglycan; IIH6 and VIA4-1 and core α-dystroglycan; GT20ADG. A) Immunohistochemical analysis of transverse sections of skeletal muscle from a dystroglycanopathy patient and a control normal muscle. The hallmark of dystroglycanopathy muscle is a specific reduction of immunoreactivity to either IIH6 or VIA-4 yet the retention of immunoreactivity to β-

dystroglycan and GT20ADG. B) Immunoblot analysis of WGA-enriched total muscle from control normal muscle (C) and a dystroglycanopathy patient (P). The molecular weight of β-dystroglycan is unchanged on western blot analysis yet a dramatic reduction in molecular weight of α-dystroglycan is seen using the GT20ADG antibody due to hypoglycosylation. Primary sequence analysis of α-dystroglycan predicts a molecular mass of 72 kDa however α-dystroglycan in skeletal muscle migrates to 156 kDa.

1.7 PERSPECTIVE

Despite the major progress made over the last 2 decades in identifying and mapping various forms of CMD, there are large gaps in our cumulative knowledge. The CMDs are clearly a very heterogeneous group and it is apparent that a large proportion of patients remain for whom no definitive diagnosis can be made. Some of these will have phenotypes that evolve allowing a diagnosis to be made in later years. Other patients fall within a pathological subgroup (for instance dystroglycanopathy) but have no identifiable gene defect. Others appear to fulfil the general clinical and pathological criteria for CMD but remain a diagnostic mystery. This suggests that some genes involved in congenital muscular dystrophy are yet to be discovered.

Establishing a correct diagnosis has never been so important. On a basic level, giving a name to a condition is often helpful to family and carers especially when negotiating complex medical and social arrangements. A diagnosis also allows accurate genetic counselling and in relevant cases may be necessary for prenatal testing. Although historically muscular dystrophies have been untreatable, supportive therapies are increasingly available and an accurate diagnosis ensures that disease complications can be anticipated and appropriate intervention established. In the last few years, advances in our understanding of the CMDs have also facilitated the pathway to therapeutic trials for these rare disorders. A confirmed genetic diagnosis is a prerequisite for enrolment in many clinical trials.

Although a proportion of CMD cases are easily diagnosed and run a predictable course, for many the path to diagnosis and then onward disease progression is less straightforward. For these families, the use of alternative investigative strategies such as muscle or brain MRI is often helpful and may allow insight where traditional diagnostic methods have failed. Even in those cases where the diagnosis is known, accurate documentation of complications and disease progression helps establish natural history data, which in turn is useful when monitoring the effect of therapeutic interventions.

CHAPTER 2: DIAGNOSTIC OUTCOMES OF REFERRALS TO UK CONGENITAL MUSCULAR DYSTROPHY SERVICE 2001-2008.

2.1 INTRODUCTION

The Dubowitz Neuromuscular Centre (DNC) was commissioned in 2001 by the UK Department of Health National Commissioning Group (NCG) to provide a national comprehensive service for Congenital Muscular Dystrophy (CMD) including the assessment, investigation and management of children with these rare disorders. The NCG service can be accessed by the referring clinical team at various levels, including clinical review of patient, assessment of muscle biopsy or molecular genetic analysis. The centre is freely available to any UK patient. The centre moved to its current location in Great Ormond Street Hospital in 2008.

2.1.1 Heterogeneity And Differential Diagnosis Of Congenital Muscular Dystrophy

Congenital muscular dystrophies are a heterogeneous group of disorders. They present in a number of ways although most commonly with muscle weakness and hypotonia in the first months of life; contractures are common and serum creatine kinase (CK) frequently elevated. Other clinical features include contractures, muscle wasting or hypertrophy and abnormalities of eye, skin and brain. Respiratory and cardiac abnormalities are also common.

There are at least 13 genetically distinct forms of CMD reported (table 1 and 2). In addition there is a further group of as yet undefined patients in whom all known forms can be excluded. A proportion of these cases are affected by CMD variants that are clinically and pathologically undistinguishable from genetically defined forms, while others, despite their clinical and/ or pathological similarities to CMD, clearly belong to a different group of conditions. Typical examples of the latter are myotonic dystrophy, some congenital myopathy variants and congenital myasthenic syndromes (2, 221). The diagnostic challenge presented by CMD and the frequently occurring differential diagnoses seen in our department has been reported by Dr A Klein and myself (222).

Skeletal muscle biopsy is an important part of the diagnostic workup of children with suspected CMD. Muscle biopsy findings in affected individuals range from mild to overtly dystrophic, depending on the muscle biopsied and the age at biopsy. What constitutes dystrophic changes in skeletal muscle is also subject to debate. For the purpose of this report, dystrophic change is defined as muscle with fibrosis and necrosis. If necrosis is not observed then muscle fibre regeneration must be present

(CA Sewry, personal communication)(4). Immunohistochemical studies of muscle are sometimes in themselves diagnostic, particularly in the case of MDC1A associated with complete merosin deficiency. In other conditions, notably collagen VI related myopathy and dystroglycanopathy, a proportion of patients will have clear cut diagnostic immunohistochemical abnormality whilst in others the findings may be subtle and inconclusive.

2.1.2 Incidence of Congenital Muscular Dystrophy

Few studies have looked at the incidence of CMD but from the reports available the incidence has been estimated at between 4.7 x 10^{-5} live births in the north of Italy and a point prevalence of 2.5 x10^{-5} in Western Sweden (1, 223). It is clear that the prevalence of different subtypes of CMD varies in different populations, often due to founder mutations. MDC1A has historically been thought of as the most common form of CMD with estimates suggesting that it accounts for between 30 and 50% of cases (2, 221). The relatively high number of reports of MDC1A is probably in part a reflection of its historically early identification, its homogenous, usually early presentation and the relative ease of pathological and genetic diagnosis. In Japan however, Fukuyama CMD (FCMD) is the most common form of CMD with an incidence estimated at 1.92-3.68/100000 births (224). This is largely due to a founder retroposon insertion mutation in the *FKTN* gene, present in the majority of Japanese FCMD patients. A number of reports have documented the frequency of this founder mutation in Japan with one study revealing its presence in 87% of FCMD chromosomes and suggesting a carrier frequency in the Japanese population of 1/88. FCMD is rarely reported outside of Japan although similar disorders with mutations in *FKTN* are increasingly being identified (127, 225-227). Okada et al have subsequently reported collagen VI deficiency as the second most common cause of CMD in the Japanese population(228). A recent Australian report of 101 CMD biopsies identified dystroglycanopathies as the most prevalent form in that population (25% of cohort) followed by collagen VI related myopathies (12%) and then MDC1A (8%) with a definitive genetic diagnosis achieved in 24% of cases (229). In 2009, Norwood et al reported a CMD combined prevalence of 0.76/100000 in the Northern England cohort studied with MDC1A comprising 0.6/100000 , UCMD 0.13/100000 and BM 0.77/100000 (230).

Here I review the diagnostic outcome of 214 UK patients clinically assessed in our department between 2001 and 2008 with a view to describing the general activity of

the service and the frequency of the various disorders encountered in our patient population.

2.2 MATERIALS AND METHODS.

The UK referrals to the Dubowitz Neuromuscular Centre between April 2001 and January 2008 as 'possible CMD' were retrospectively reviewed. Only patients who had been clinically assessed by us at the centre and from whom a muscle biopsy was available for the diagnostic studies were included. Patients where biopsy sample was unavailable or insufficient for analysis have been excluded. Where more than one member of a family was seen, only one member of the family has been included for the purpose of analysis (the patient with the most information available).

Clinical notes, genetic tests and muscle biopsy reports were reviewed for all patients fulfilling the criteria. Diagnostic and biopsy information was gathered firstly for all referrals, which however consisted of a considerable number of patients not affected by CMD, and then subsequently for patients who fulfilled the tighter inclusion criteria for CMD. This included dystrophic or myopathic muscle biopsy with no structural features suggestive of alternative diagnosis, presentation before the age of 2 years with hypotonia, weakness, contractures, delayed motor milestones, elevation of serum CK in several variants, and characteristic eye or brain abnormality. Patients in whom a diagnosis of a condition other than CMD was made were excluded from this 'CMD subgroup' analysis.

Diagnostic standards were as for Dubowitz and Sewry 2007 (CMD and congenital myopathy)(4), Godfrey et al 2007 (dystroglycanopathy)(231), Gerenmayeh et al 2010 (MDC1A)(34), Nadeau et al 2009 (collagen VI)(232) and Kinali et al 2008 (congenital myasthenia)(233). All biopsy samples from patients referred with suspected CMD underwent a panel of routine investigation including histological, histochemical and immunohistochemical analysis except for those where sample size limited testing. For further information on standard departmental testing procedure see
http://www.ich.ucl.ac.uk/gosh/clinicalservices/neuromuscular_services/.

Genetic testing was directed by biopsy and clinical findings and often supported by muscle MRI investigation (40). Molecular genetic analysis of CMD and congenital

myopathy genes was provided by Guys and St Thomas' Trust, London (part of CMD NCG service), the NCG Referral Centre for LGMD at the Institute of Human Genetics at Newcastle University and NCG funded Oxford Congenital Myasthenic Syndrome Service. Key references for the listed diseases and their diagnostic workup can be found at: http://www.musclegenetable.org/.

2.3 RESULTS

2.3.1 Total Cohort Analysis 'All Referrals'

Muscle biopsy samples were received from 415 patients. Of these, 218 patients were clinically assessed in the Centre. Four siblings were excluded leaving a 'total referral' number of 214 families included in subsequent analysis.

From these 214 patients, the average age at biopsy assessment was 6.2 years (range 1 day - 37.2 years). The referrals represent a very mixed group in terms of ethnicity reflecting the London and UK population. Patients were referred from throughout the UK; 74/ 214 were referred from the Greater London area and 140/214 were UK patients referred from outside Greater London with 55/101 non-Greater London postcodes represented in the sample (Figure 8).

A genetic diagnosis was reached in 83/214 (39%). A further 22 patients (10%) could be confidently assigned to a pathological subgroup on the basis of characteristic muscle biopsy findings. In total, 105 patients had a diagnosis molecularly confirmed or pathologically refined. Of these 105 patients, 64 were affected by a form of CMD (Table 3 and 4). There are a further 23 patients in whom a clinical diagnosis is strongly suspected but a definitive genetic or pathological diagnosis could not be reached.

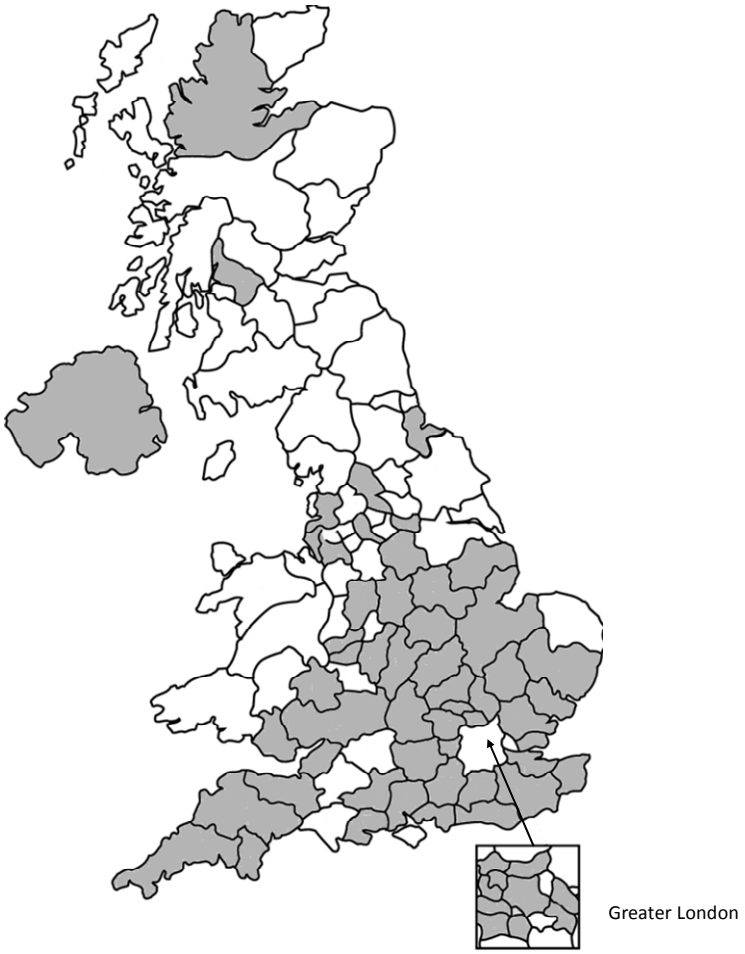


Figure 8: UK postcode map showing regions from which referrals were received (shaded areas).

Our referral group is comprised of geographically and ethnically diverse patients from most regions of the UK but predominantly England.

DIAGNOSIS	NUMBER	PHENOTYPE	NUMBER	GENE
Congenital Muscular Dystrophy	57	Collagen VI- related (BM =5) (UCMD =17)	22	*Collagen VI* =22
		Dystroglycanopathy	16	*POMT1* =2 *POMT2* =3 *POMGNT1* =5 *FKRP* =5 *LARGE* =1
		EDMD2*	4	*LMNA* =4
		MDC1A	12	*LAMA2* =12
		RSMD1	3	*SEPN1* =3
Congenital Myopathy	11	CCD	4	*RYR1* = 4
		Myotubular Myopathy	3	*MTM1* =3
		Nemaline Myopathy	3	*ACTA1* = 3
		CFTD	1	*TPM3* = 1
Limb Girdle Muscular Dystrophy	4	Dystroglycanopathy	4	*FKRP* = 4
Congenital Myasthenia	8	Congenital Myasthenia	8	*CHRNA1* =2 *COLQ* = 1 *DOK7* =3 *RAPSYN* =1 *CHRNG* =1
Other	3	EDS	1	*Collagen 3a1* =1
		RETT Syndrome	1	*MECP2* =1
		Chromosomal	1	Chromosomal =1
TOTAL	83			

Table 3. Distribution of genetic diagnoses (n=83) in the 'all referrals' group (n=214).

BM; Bethlem Myopathy, UCMD; Ullrich congenital muscular dystrophy, EDMD2; autosomal dominant Emery- Dreifuss muscular dystrophy, MDC1A; merosin deficient CMD, RSMD1; rigid spine muscular dystrophy, CCD; central core disease, CFTD; congenital fibre type disproportion, EDS; Ehlers- Danlos syndrome.

*Included in CMD group because of early presentation in these cases.

DIAGNOSTIC GROUP	NUMBER	PHENOTYPE	NUMBER
Congenital Muscular Dystrophy	7	Collagen VI related (BM = 2) (UCMD = 2) Dystroglycanopathy	4 3
Congenital Myopathy	13	Core Myopathy Centronuclear Myopathy Myofibrillar Myopathy Nemaline Myopathy	3 4 1 5
Other	2	Mitochondrial Pompe Disease	1 1
TOTAL	22		

Table 4. Distribution of pathological 'diagnoses' (n=22) in the 'all referrals' group (n=214).

BM; Bethlem myopathy, UCMD; Ullrich congenital muscular dystrophy.

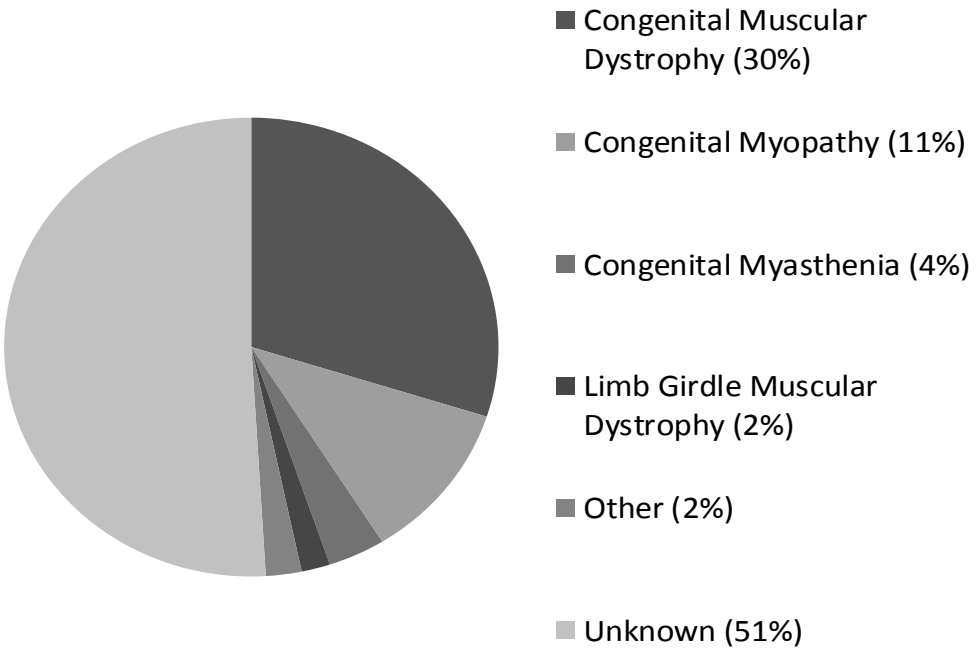


Figure 9. Distribution of confirmed diagnoses (molecular or pathological) in the 'all referrals' group of patients (n=214).

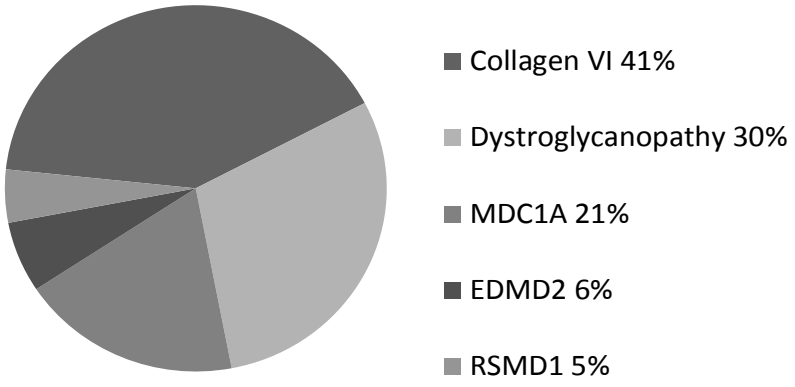


Figure 10. Relative frequency of confirmed CMD diagnoses (n=64) within 'all referrals' group.

MDC1A; merosin deficient CMD, EDMD2; autosomal dominant Emery- Dreifuss muscular dystrophy, RSMD1; rigid spine muscular dystrophy.

2.3.2 CMD Subgroup Analysis

For this analysis, patients were chosen from the above 214 who fulfilled the following criteria:

1. Dystrophic, myopathic or minimal change muscle biopsy. Patients were excluded from this subgroup analysis if biopsy showed additional 'structural' change (e.g. rods) suggesting an alternative diagnosis. Patients with cores were included, as long as RYR1 mutation had been excluded, as they have been reported in patients with RSMD1 and also more rarely in EDMD2 and UCMD (CA Sewry and F Muntoni personal communication).
2. Presentation before 2 years of age with hypotonia, weakness, contractures, delayed motor milestones, raised CK or characteristic eye or brain abnormality.
3. Non CMD diagnoses (or patients in whom alternative diagnosis is strongly suspected) excluded.

One hundred and sixteen patients fulfilled all 3 criteria. Average age at biopsy assessment was 6.4 years (range 8 days -28.4 years). 37/116 patients were referred from Greater London, 79/116 were referred from outside Greater London.
A molecular diagnosis was reached in 58/116 (46%) patients and a pathological subgroup could be assigned to another 5 patients (4%) (Table 5 and Figure 11).

Of the 57 patients in the CMD subgroup with clearly dystrophic biopsies, a molecular diagnosis was made in 43 (75%) and a pathological subgroup could be assigned in a further 3 patients.

DIAGNOSIS	CMD SUBGROUP	NUMBER	GENE
Genetic (n=53)	Collagen VI related (BM =5) (UCMD =17)	22	*Collagen VI* =22
	Dystroglycanopathy	14	*POMT1* =2 *POMT2* =3 *POMGNT1* =4 *FKRP* =4 *LARGE* =1
	EDMD2	4	*LMNA* =4
	MDC1A	12	*LAMA2* =12
	RSMD1	1	*SEPN1* =1
Pathological (n=5)	Collagen VI related	2	-
	Dystroglycanopathy	3	-
Unknown (n=58)	-	58	-
Total		116	

Table 5. Distribution of the genetic and pathological diagnoses in the 'CMD subgroup'.

BM; Bethlem Myopathy, UCMD; Ullrich congenital muscular dystrophy, EDMD2; autosomal dominant Emery- Dreifuss muscular dystrophy, MDC1A; merosin deficient CMD, RSMD1; rigid spine muscular dystrophy.

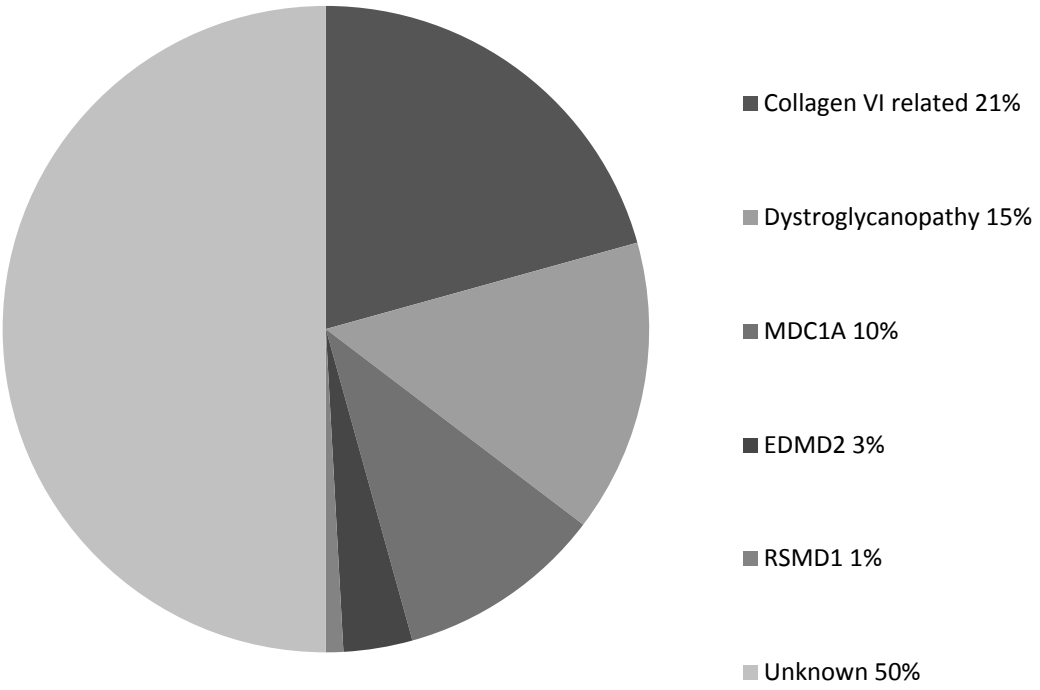


Figure 11. Graph showing distribution of diagnoses (n=58) in the 'CMD subgroup'.

MDC1A; merosin deficient CMD, EDMD2; autosomal dominant Emery- Dreifuss muscular dystrophy,RSMD1; rigid spine muscular dystrophy.

2.3.3 Skin Biopsies

A CMD diagnosis was also made from analysis of skin biopsy in 5 out of 6 patients in whom this was the only biopsy material received. These were all Collagen VI related disorders in which the expression of collagen VI was reduced.

2.3.4 Overseas Patients

In addition to the UK patients reported, we also received muscle biopsy samples from 34 patients from abroad, mostly from the Middle East and Europe. Nineteen of these patients were reviewed by the department and a diagnosis was reached in 13/19. In 11 patients a genetic diagnosis was confirmed, 8 of these were dystroglycanopathy patients, 2 had MDC1A and a single patient had a core myopathy. The remaining 2 patients were diagnosed on the basis of their pathology with a dystroglycanopathy and a Collagen VI related disorder. These patients have been excluded from further analysis here as I wanted to present the UK referral data and felt that the overseas referrals are overly biased towards our research interests, in particular the dystroglycanopathies.

2.4 DISCUSSION

I present here the diagnostic outcome in a large cohort of UK patients referred to the CMD NCG centre for clinical review and muscle biopsy analysis between 2001 and 2008. The 214 patients reported represent 52% of the UK patients with biopsy samples assessed in the NCG Centre during this time.

Examining the ‘all referral’ group, a genetic or pathological diagnosis was made in 49% of patients (105/214) referred with ‘possible CMD’. A molecular diagnosis was established in 39% of patients (83/214). A total of 64 patients in the “all referral” group (30%) had a confirmed (genetic or pathological) diagnosis of CMD.

One hundred and sixteen patients fulfilled the stricter ‘CMD’ inclusion criteria. A diagnosis of CMD was made in 50% of these patients (58/116) and was genetically confirmed in 53/116 (46%). Within this group, collagen VI related myopathy was the most common diagnosis with 24/116 (21%), followed by dystroglycanopathies with 17/116 (15%). A diagnosis of MDC1A was made in 12/116 (10%), EDMD2 in 4/116 (3%) and RSMD1 in 1/116 (1%). Two further genetically proven cases of RSMD1 were

excluded from this 'CMD' subgroup analysis as they presented after 2 years of age. I found no cases of Integrin α7 deficiency in our population. Diagnoses were not determined in 58 cases (50%). It should be noted that we documented a further 5 cases of collagen VI related disorders confirmed from analysis of skin biopsy alone in patients not assessed at our centre and therefore not included in this report, reinforcing this as the most prevalent CMD variant in our referral population.

The inclusion of biopsies with cores in the CMD subgroup analysis has almost certainly led to the presence of a number of undiagnosed congenital myopathy cases in this sample. However, as cores may be observed in RSMD1 and in some cases of EDMD2 and UCMD, we felt that including these biopsies was appropriate, if RYR1 gene mutation had been ruled out. In the CMD subgroup, 6 samples were included with myopathic features and cores, only one of these had a confirmed diagnosis (SEPN1 mutation).

Our results contrast those of a recent Australian study by Peat et al where a specific histopathological diagnosis could be allocated in 49% of 101 CMD cases and a definitive genetic diagnosis could be made in 24% (229). It should be noted that patients with biopsies containing cores were excluded in their study therefore direct comparison with our data should be done with care. Even taking this into account, our definitive (genetic) diagnosis rate is almost twice as high (46% v 24%) when comparing our data to the Australian CMD cohort. The reasons for the differences in the diagnostic pick up rate are most likely a reflection of the fact that all of the patients in this study were clinically assessed by the NCG service and that many patients also had additional supportive investigations including analysis of collagen VI in fibroblasts and muscle MRI imaging to help direct genetic investigations. We have previously reported that muscle MRI is a particularly useful diagnostic adjunct in investigating these disorders with a high specificity and sensitivity for different subtypes of CMD (40). We have found MRI to be especially useful in the case of collagen VI related disorders where we have found it to be a more reliable indicator of collagen VI pathology than fibroblast analysis. Indeed abnormal production of collagen VI in fibroblast cultures is a sensitive indicator but not specific for collagen VI abnormality (Francesco Muntoni-unpublished data)

In the Australian study, dystroglycanopathy was found to be the most common group representing 25% of cases, with collagen VI abnormalities found in 12% (229). Studies from Japan reveal FCMD (a dystroglycanopathy) to be the most prevalent form of CMD in the Japanese population (49.2% of CMD cases in one series) but this is due to a

FKTN founder mutation. The second most common form of CMD encountered in the Japanese population is Collagen VI deficiency with an estimated frequency of 7.2% in their CMD cohort. Interestingly, a 2009 paper from the North of England (230) found MDC1A to be the most prevalent CMD disorder (0.6/100000) and much more common than a CMD presentation of dystroglycanopathy (0.03/100000), UCMD (0.13/100000) and RSMD1 (0.13/100000). However, mutations in FKRP are more frequently seen in their LGMD cohort (0.43/100000). In addition, BM has an estimated prevalence of 0.77/100000 in their series but as there is no information about the age of presentation of these patients, comparison between the collagen VI related disorders in that study and ours is of limited value.

Analysis of our data suggests that MDC1A, in various series of patients reported to be the most common form of CMD, is not the most frequent diagnosis in our population, as both Collagen VI related disorders and dystroglycanopathy are more prevalent. This may be a reflection of improved diagnostic assays and greater awareness of the clinical and pathological presentation of other CMD forms; regional variation in the prevalence of CMD subtypes may also be implicated.

It is also of interest to note the large number of non CMD diagnoses made in the 'all referrals' group, reflecting the importance of an integrated approach to the diagnosis of these rare and heterogeneous conditions, which includes clinical examination, pathological diagnosis and muscle imaging directing the genetic testing. These results also confirm our previous observations of conditions resembling CMD, which include several congenital myopathies and congenital myasthenias which may show significant clinical and pathological overlap with CMD (222). Indeed, the NCG service has now expanded to include assessment of congenital myopathy cases as a consequence of the diagnostic difficulties presented by these groups of conditions.

Despite our integrated approach, we were unable to reach a diagnosis in just over half of the 'all referrals' group. These undiagnosed cases are likely to comprise a number of non CMD diagnoses, a number who have an as yet undefined form of CMD caused by mutations in a novel gene and also some patients with a defined form of CMD in whom we are unable to confirm this pathologically or genetically. Although the sensitivity of genetic testing has steadily improved, large heterozygous deletions or duplications are likely to be missed using present techniques. In some patients variants of unknown significance will have been detected that cannot be proven to be pathogenic using current methods. Some of the undiagnosed patients will eventually 'reveal' their diagnosis as their symptomatology and histopathology evolves. For example, recent

studies highlight the mild initial pathological features in UCMD (234), and the selectivity of muscle involvement which characterises many of these conditions (40). In others, a diagnosis may be made as a result of new technology, in particular the emergence of the NMD chip for block testing neuromuscular disorders in affected individuals. The results of the first round of screening for the NMD chip are pending and the first few large rearrangements have been detected using this technique. (http://www.nmd-chip.eu/about). Although the NMD chip as well as recent advances in next generation sequencing technologies are very exciting advances in the field of CMD testing, the quantity of data generated by such methods and in turn the accurate interpretation of results is likely to present its own set of challenges.

With regard to the clinical presentation of the CMDs; although MDC1A is usually seen as a classical presentation of severe early weakness, hypotonia and later characteristic features on brain MRI, the collagen VI related disorders and dystroglycanopathies are frequently more challenging to diagnose due to their often milder phenotypes. In both groups and also in EDMD2 and RSMD1, the classification lines between CMD, myopathy and LGMD are frequently blurred. This is again reflected in the number of cases of LGMD and myopathy seen in our 'all referrals' series of patients.

The geographically diverse nature of our referral population unfortunately precludes any formal estimation of prevalence or incidence for the different CMD subtypes, none the less, the information gathered here I feel is an accurate reflection of clinical activity in the CMD NCG service. It is of interest to note that over 50% of patients from outside greater London were represented in our patient population highlighting the nationwide uptake of services.

In summary, the data presented in this study represents the largest series of UK CMD referral data and diagnostic outcome reported. It reveals Collagen VI related to be the most common form of CMD in our patient group, accounting for 21% of referrals that fulfil the CMD inclusion criteria. Patients with dystroglycanopathy follow closely behind accounting for 15% with MDC1A seen in 10%. There remain a large proportion of cases (50%) that fulfil the clinical and pathological criteria for CMD, in whom no diagnosis can be made. This suggests that further CMD subtypes and genes are yet to be discovered.

CHAPTER 3: REFINING THE GENOTYPE-PHENOTYPE RELATIONSHIP IN A LARGE COHORT OF DYSTROGLYCANOPATHY PATIENTS.

3.1 GENOTYPE PHENOTYPE CORRELATION IN A LARGE COHORT OF DYSTROGLYCANOPATHY PATIENTS

3.1.1 Introduction

Genotype-phenotype correlation in the dystroglycanopathies: a historical perspective.

Dystroglycanopathy phenotypes have been recognised for many decades, although it has only been in recent years that the pathological and molecular basic for these disorders has begun to be unravelled. Walker Warburg Syndrome (WWS) is named after Walker who first identified the type 2 lissencephaly associated with the condition and Warburg who reported several more cases, appreciating the autosomal recessive inheritance (235, 236). Eye abnormalities were identified as a concurrent feature by Whitley *et al*. in 1983 and associated the myopathy was identified in 1984 (237, 238). WWS had also been reported by a number of other investigators and has historically been known by several other names including HARD +/- E (**H**ydrocephalus, **A**gyria, **R**etinal **D**ysplasia +/- **E**ncephalocele) and COD-MD (**C**erebro-**O**culo-**D**ysplasia/ **M**uscular **D**ystrophy)(239, 240). In 1989, Dobyns and co-workers reported a large series of new patients and reviewed the existing literature leading to the establishment of diagnostic criteria and the appreciation that many of the previously reported conditions were in fact the same disease entity (241). They proposed 4 key diagnostic criteria including type 2 lissencephaly (cobblestone complex), retinal abnormality, CMD and cerebellar malformation. A number of other clinical features were also reported but were not considered essential for diagnosis.

Muscle Eye Brain disease (MEB) was first identified in the Finnish population. The key features of CMD, ophthalmic abnormality and hydrocephalus were originally reported in 1977(242). Subsequent reports, again of Finnish patients, followed in the 1980's, highlighting the extent of the brain abnormality, including cortical migration abnormality consistent with type 2 lissencephaly, but not as severe as that seen in WWS(243, 244).

Fukuyama Congenital Muscular Dystrophy (FCMD) was first reported in 1960 by Fukuyama *et al*. who identified 15 cases in Japan with CMD and associated structural brain abnormalities (212). By 1981 more than 200 cases had been recognised in Japan although it had not been seen outside of this population. Key features seen in FCMD include mental retardation, seizures, type 2 lissencephaly, cerebral and cerebellar polymicrogyria, hydrocephalus and hypoplasia of the corticospinal tracts (245, 246).

Lissencephaly type 2 is a feature common to all 3 disorders. Lissencephaly describes a smooth appearance of the cerebral cortex. It is caused by defective neuronal migration during development of the cerebral cortex and is found in a variety of disorders. Lissencephaly type 2 however, is an unusual finding and is caused by the overmigration of neurones through the basal lamina resulting in a nodular 'cobblestone' appearance to the cerebral surface (247). This is covered in more detail in section 4.1.1.

The common finding of type 2 lissencephaly in WWS, MEB and FCMD led researchers to consider the relationship between these conditions. Indeed Dobyns *et al.* postulated that WWS and MEB were in fact the same entity, whilst Santavuori *et al*. thought that there were enough differences between the 2 disorders to consider them separate disorders (241, 248, 249). In particular, Santavuori considered that MEB tended to have milder cerebellar malformations and pachygyria +/- polymicrogyria rather than large areas of lissencephaly. The eye abnormalities in MEB were often juvenile onset and progressive rather than the congenital abnormalities frequently observed in WWS. Both research groups agreed that FCMD, although similar in many aspects, was a different condition to both WWS and MEB. In particular, cerebellar and eye abnormalities were not a prominent feature and the clinical course was generally less severe. A later study by Yoshioka *et al.* (250) highlighted the differences between FCMD and MEB/ WWS but also revealed a clinical overlap between the more severe FCMD and the milder WWS/ MEB cases. The lack of genetic and molecular data at this time prevented any definitive conclusions being drawn about how these conditions related to each other.

The first major advance in understanding the relationship between these three conditions came with the identification of the FCMD locus and subsequent reports of mutations in the *Fukutin* (*FKTN*) gene in 1998 (127, 215). A common founder retrotransposal insertion was found in more than 80% of FCMD chromosomes, accounting for the high prevalence of FCMD in the Japanese population. This was followed in 1999 with the localisation of the MEB locus 1p32-p34 and in 2001 by the report of *protein O-linked mannose β1,2-N-acetlyglucosaminyltransferase 1* (*POMGnT1)* mutations in Finnish and Turkish patients with MEB (129, 209). Cormand *et al*. subsequently analysed a large cohort of MEB and WWS patients and effectively excluded linkage to 1p32-p34 from all but 1 patient with diagnostic criteria compatible with WWS. Conversely all patients except 1 Swedish girl with features consistent with MEB linked to the locus. The first gene identified in patients with WWS was reported a

few years later when *Protein O-mannosyltransferase* (*POMT1)* was found to be mutated in 20% of a WWS patient cohort (130). This provided strong initial evidence for the genetic distinction between MEB and WWS although did not provide an explanation for the similarity observed between the conditions (206).

The recognition of the 'dystroglycanopathies' as a specific pathological subgroup defined by hypoglycosylation of ADG marked a key turning point and heralded the discovery of mutations in three further genes, *FKRP*, *LARGE* and *POMT2*. All were good candidate genes because of homology to existing genes or in the case of *LARGE*, involvement in the Large^{Myd} mouse.

In 2009, after this research was completed, a further gene, *Dolichyl-phosphate mannosyltransferase polypeptide 3 (*DPM3) was identified. Mutations in this gene have been shown to give rise to CDG1o, a condition with features overlapping that of a dystroglycanopathy and congenital disorder of glycosylation. This gene has been excluded from the remaining discussion in this section as its discovery postdates the research, hence all references to the '6 known genes' refer to *POMT1, POMT2, POMGNT1, FKRP, FKTN* and *LARGE.*

As more patients were described with mutations in the 6 known genes, it became increasingly apparent that the phenotype associated with mutations in a specific gene could be very variable, demonstrated best by the wide spectrum of severity seen in patients with mutations in *FKRP,* where patients may be severely affected by WWS, mildly affected by LGMD2I or exhibit a phenotype in between the 2 extremes. In addition to the WWS phenotype, *POMT1* mutations were subsequently described in patients with CMD and microcephaly with mild mental retardation (termed CMD-mental retardation) (251) and also in patients with LGMD with microcephaly and mental retardation (LGMD2K)(125, 252). *FKTN* mutations were also isolated in non Japanese patients with WWS and then subsequently in patients with a predominant dilated cardiomyopathy phenotype with minimal muscular involvement (CMD1X)(32, 207, 226).

Identifying the molecular genetic basis for the dystroglycanopathy phenotypes, far from clarifying the question of whether to lump or split this group of conditions, clouded the situation further. In brief, most phenotypes could be caused by more than one gene and conversely some genes could cause more than one phenotype. The phenotypes

and associated genes known in 2006, at the start of this work, are summarised in Table 6. Due to the numerous different names given to the milder phenotypes, for clarity, these have been simplified into CMD or LGMD with and without mental retardation, with other names referenced as necessary.

	Phenotype								
Gene	WWS	MEB	FCMD	CMD-CRB	CMD - MR	CMD – no MR	LGMD -MR	LGMD – no MR	CMD 1X
POMT1	•(130, 251, 253)				•(130, 251, 253)		•(125, 252)[a]		
POMT2	•(132)	•(210)							
POMGNT1		•(129, 254)							
FKTN	•(208, 226)		•(127, 255)					•(126)	•(32)
FKRP	•(207)	•(207)		•(211)	•(211)	•(128)[b]		•(192)[c]	
LARGE					•(131)[d]				

Table 6. Summary of the main dystroglycanopathy phenotypes, associated gene defects and key references prior to commencement of our study. The phenotype initially associated with each gene is shaded in grey.

WWS; Walker Warburg Syndrome, MEB; Muscle Eye Brain Disease, FCMD; Fukuyama CMD, CMD-CRB; CMD with cerebellar involvement, CMD - MR; CMD with mental retardation, CMD –no MR; CMD without mental retardation, LGMD - MR; LGMD with mental retardation, LGMD –no MR; LGMD without mental retardation, CMD1X; dilated cardiomyopathy 1X.

[a] Phenotype also reported in literature as LGMD2K, [b] Phenotype also reported in literature as MDC1C, [c] Phenotype also reported in the literature as LGMD2I, [d] Phenotype also reported in the literature as MDC1D.

Whilst a lot of information was being published on this group of disorders, the relative rarity of these conditions meant that data was not available regarding the extent to which molecular and phenotypic overlap occurred and also the proportion of patients for which all known genes could be excluded. Our department found itself in the fortunate position of being able to address this issue, having accumulated a significant number of DNA and muscle biopsy samples from patients with dystroglycanopathies, due a to prior research interest in this field.

All previous studies had been conducted on a small number of families or individuals. This causes inevitable difficulties in applying mutation detection rates to the general population. In addition such reports make it difficult to establish whether the described clinical spectrum is truly representative of the phenotypic variability and also how common the originally described core phenotypes are for each of these genes. In order to address these points, we systematically screened a large population of patients with a secondary dystroglycanopathy phenotype for mutations in the associated genes. As the spectrum of phenotypes secondary to *FKRP* involvement has been previously reported by us and others, we studied ninety seven patients in whom involvement of this gene had been excluded before proceeding with analysis of the 5 remaining genes. Patients in whom findings of particular interest were noted, have been investigated further with functional studies and are reported in detail.

This comprehensive study redefines the clinical spectrum associated with each of the glycosyltransferase genes studied, identifies the frequency of individual gene defects and suggests that the majority of patients with a dystroglycanopathy do not have mutations in any of the known genes.

3.1.2 Patients and Methods

Patients.

The cohort consisted of 92 unrelated individuals including a large group of patients from Australia (27 patients) and Turkey (16 patients). The majority of the remaining patients were recruited via the Hammersmith Hospital National Commissioning Group (NCG) service and included DNA from individuals referred from across the UK and Europe with a few samples from further afield. Mutations in *FKRP* had previously been excluded in all cases(128). Criteria for inclusion included hypoglycosylation of ADG at the sarcolemma of skeletal muscle demonstrated by immunolabelling (4, 256). Eighty patients met these criteria whilst in the remaining 12 cases there was no muscle available for ADG studies. In the remaining 12 patients, inclusion in the cohort was justified due to their clinical phenotype being highly suggestive of a dystroglycanopathy and consisted of children with CMD, elevated serum CK and brain MRI evocative of type 2 lissencephaly. Common CMD and LGMD conditions (dystrophinopathy, LGMDs such as sarcoglycanopathies, calpainopathy and dysferlinopathy, merosin deficient CMD and collagen VI deficiency) were excluded in all patients with available muscle biopsy by standard immunocytochemical and/or Western blotting analysis (4). Clinical data was collated and patients were divided into defined phenotypic categories. This study was approved by Hammersmith Hospital Ethics Committee REC 2000:/5802. Control samples were from a cohort of parental bloods of White European ethnicity following ethical approval by the Hammersmith and Queen Charlotte's and Chelsea Trust Research Ethics Committee (2001/6029).

Molecular Genetics.

Mutation screening was performed by Caroline Godfrey whilst working at the DNA laboratory, GSTS Pathology at Guy's Hospital.

Genomic DNA was extracted in the referring centre's laboratory using standard protocols. All mutation scanning was performed in the DNA laboratory at Guy's Hospital. The complete coding regions, including intron / exon boundaries of *POMT1*, *POMT2*, *POMGNT1*, *FKTN* and *LARGE* were amplified by PCR. Single nucleotide polymorphisms (SNP) within the primer binding sites were avoided using the Diagnostic SNP Check software (ngrl.man.ac.uk/SNPCheck). Amplicons were screened for mutations using a combination of uni-directional sequencing using standard dideoxynucleotide methodology and heteroduplex analysis as previously

described (257). Where available, parental DNA was studied once a sequence alteration was identified in the proband. In two families, further segregation analysis was carried out to investigate the potential pathogenicity of unclassified variants. In families where a *de novo* mutation was suspected, paternity was confirmed using 11 STR markers. Mutation nomenclature based on the following GeneBank Accession numbers; *POMT1*; NM_007171.2, *POMT2*; NM_013382.3, *POMGnT1*; NM_017739.1, *FKTN*; NM_006731.1 and *LARGE*; NM_133642.2, with nucleotide number 1 corresponding to the first base of the translation initiation codon.

3.1.3 Results

Clinical Findings

Patients were classified as having either a CMD or LGMD phenotype and further subdivided according to the degree of structural and functional brain involvement. CMD was defined as onset of weakness prenatally or within the first 6 months of life. LGMD was defined by later onset weakness, specifically after having acquired ambulation. The cohort consisted of a total of 64 patients with CMD and 25 patients with LGMD, a total of 59 patients had brain involvement. In three patients the clinical information was insufficient to be able to assign a phenotypic category. Patients were divided into 1 of 7 phenotypic categories summarised below;

1) **WWS (and WWS-like):** Onset prenatally or at birth. Patients assigned to this category had severe structural brain abnormalities including complete agyria or severe lissencephaly with only rudimentary cortical folding, marked hydrocephalus, severe cerebellar involvement and complete or partial absence of the corpus callosum. Eye abnormalities including congenital cataracts, microphthalmia and buphthalmos were common. When MRI evidence was not available, death before 1 year of age was taken as suggestive of this category if other clinical findings were supportive (206). Motor development was typically absent in these patients. Five patients were assigned to this group.

2) **MEB/ FCMD-like:** These categories were merged due to the overlapping phenotypic features. Included in this group were CMD with brain abnormality less severe than that seen with WWS. MRI findings include pachygyria with preferential frontoparietal involvement, polymicrogyria, cerebellar hypoplasia and dysplasia and frequent flattening of the pons and brainstem. Eye abnormalities are often seen and include congenital glaucoma, progressive myopia, retinal atrophy and juvenile cataracts.

Individuals may, rarely, acquire the ability to walk although this is delayed. Rarely patients manage to learn a few spoken words. Thirty patients were assigned to this group, including one in whom the clinical information was limited.

3) **CMD-CRB (CMD with cerebellar involvement):** This category included CMD with mental retardation and cerebellar involvement on MRI scan as the only structural abnormality. Cerebellar abnormalities may include cysts, as described relatively frequently in individuals with *FKRP* gene defects (258) or cerebellar hypoplasia or dysplasia. Four patients were assigned to this group.

4) **CMD-MR (CMD with mental retardation):** CMD with mental retardation and structurally normal brain. Patients with isolated microcephaly or minor white matter changes on MRI are included in this group. Fifteen patients were assigned to this group, including two with limited clinical information.

5) **CMD-no MR (CMD with no mental retardation):** Several patients within this group have not had neuroimaging but had entirely normal intellectual function. Ten patients were assigned to this group, one with limited information.

6) **LGMD-MR (LGMD with mental retardation):** LGMD with mental retardation and structurally normal brain. Patients with minor white matter abnormalities and microcephaly were included in this group. This category would include patients with a phenotype resembling LGMD-2K (125). Five patients were assigned to this group.

7) **LGMD-No MR (LGMD with no mental retardation):** LGMD with no mental retardation. This category would include the LGMD phenotypes resembling LGMD2I and 2L (257). Twenty patients were assigned to this group, six with limited clinical information.

The division of phenotypes within the cohort is shown in Table 10. Detailed clinical information is contained in Table 7 for those patients in whom pathogenic mutations were detected.

Patient	ADG	Phenotype a	Age at onset b	CK	Motor ability c	Contractures d	Hypertrophy e	Spine f	Eyes g	Weakness h	IQ i	Microcephaly j	MRI k	Other l
1	LOW	WWS	P	4000	NS	Y	Y	Sc, RS	Poor visual attention	LL>UL	L	Y	H, CHy, WM, Lis	Gastrostomy
2	LOW	MEB-FCMD	P	3500	N/A	Y	N/A	N/A	CG	N/A	L	Y	H, BS,WM,CC,CHy	N/A
3	LOW	LGMD-MR	I	2000	W	N/A	Y	U	N/A	N/A	L	Y	Normal	N/A
4	LOW	CMD-MR	I	7800	NW 2yr	N/A	N/A	U	U	N/A	L	N/A	WM	N/A
5	LOW	LGMD-MR	I	4000	W	N	Y	U	U	N/A	L	Y	Normal	N/A
6	LOW	LGMD-MR	3Yr	8000	W		Y	N/A	U	N/A	L	Y	WM- minimal	N/A
7	LOW	CMD-MR	I	3600	St	N	Y	U	U	N/A	L	Y	Normal	N/A
8	LOW	CMD-MR	4m	18000	W	N/A	Y	RS	N/A	N/A	L	Y	WM- minimal	Choreic Movement disorder
9	LOW	MEB-FCMD	N	5500	S	Y	Y	RS, Sc	N/A	N/A	L	Y	WM, BS	N/A

Patient	ADG	Phenotype a	Age at onset b	CK	Motor ability c	Contractures d	Hypertrophy e	Spine f	Eyes g	Weakness h	IQ i	Microcephaly j	MRI k	Other l
10	LOW	MEB-FCMD	4Yr	5200	NW	N	Y	U		N/A	L	Y	Encephalocele	N/A
11	LOW	MEB-FCMD	7m	N/A	NS	Y	N	U	Hm	N/A		N/A	H, WM,CC,	N/A
12	LOW	MEB-FCMD	N	3100	NS	Y	N/A	RS		UL>LL	L	Y	WM	N/A
13	N/A	MEB-FCMD	8m		W	N/A	Y	U	My	UL>LL	L	N	WM , CDys, CC,PMG	N/A
14	LOW	MEB-FCMD	N	6000	S	Y	Y	Sc	CC	N/A	L	Y	BS,H,WM	SE, RIP age 11yr
15a	N/A	CMD-CRB	I	4700	W	Y	Y	N/A	N/A	UL>LL	L	Y	N/A	N/A
15b	N/A	CMD-CRB	I	5200	S	N/A	N/A	N/A	N/A	N/A	L	N/A	CHy	Micropenis and cryptorchidism
16	LOW	LGMD-MR	18m	1900	W	N	Y	U	N/A	N/A	L	N/A	NO MRI	RBBB on ECHO
17	LOW	MEB-FCMD	N	2000	NS	Y	Y	N/A	My	UL,LL	L	Y	CHy, H	Macroglossia
18	LOW	MEB-FCMD	I	780	NW	N	N	N/A	CG	N/A	L	N/A	BS,CC,WM,H	N/A

Patient	ADG	Phenotype a	Age at onset b	CK	Motor ability c	Contractures d	Hypertrophy e	Spine f	Eyes g	Weakness h	IQ i	Microcephaly j	MRI k	Other l
19	LOW	MEB-FCMD	P	1000	W	Y	Y	U	OA, My	N/A	L	N	WM,CC	SE, feeding difficulties
20	LOW	LGMD-no MR	12 Yr	12000	R	N	Y	U	My	LL>UL	NI	N		N/A
21	LOW	MEB-FCMD	N	1200	NONE	N	N	U	RD	N/A	L	N/A	H,WM,CC	SE, feeding difficulties.
22	LOW	MEB-FCMD	12m	2800	R	N	N/A	U	Pt, RA	N/A	L	N/A	CHy, CC, WM,H	Dyspraxia, feeding difficulties, SE
23	LOW	MEB-FCMD	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	H,CC,WM	N/A
24	N/A	WWS	N	1300	NS	N/A	N/A	U	N/A	N/A	L	N/A	CC,CHy, WM, H,Lis	N/A
25	LOW	WWS	P	5700	NONE	Y			RDy		L		H,WM,CHy, Lis	Feeding difficulties, RIP 8 weeks
26	LOW	CMD-no MR	3Yr	3200	S	N	N	U	U	G	NI	N/A	WM-MILD	Hypothyroid
27	N/A	MEB-FCMD	I	4000	S	N	Y	U	U	N/A	L	N/A	CC,WM,H	
28	LOW	WWS	N	7000	N/A	Y	Y	U	RD,Mo	N/A		N/A	WM,CHy, BS, H	Dysmorphic

Patient	ADG	Phenotype a	Age at onset b	CK	Motor ability c	Contractures d	Hypertrophy e	Spine f	Eyes g	Weakness h	IQ i	Microcephaly j	MRI k	Other l
29a	LOW	LGMD-no MR	4m	10000	W	N	Y	N/A	N/A	UL>LL	NI	N	N/A	Steroid responsive
29b	LOW	LGMD-no MR	4m	13000	W	N	Y	U	U	LL>UL	NI	N	Normal	Steroid responsive
30	LOW	LGMD-no MR	10m	60000	W	N	Y	U	U	LL>UL	NI	N	H-MILD	Steroid responsive
31a	LOW	LGMD-no MR	4yr	9000	R	Y	N/A	U	N/A	N/A	NI	N/A	N/A	N/A

Table 7. Clinical Characteristics of 33 individuals from 31 families in whom mutations were detected.

[a] WWS; Walker-Warburg Syndrome, MEB/FCMD; Muscle-Eye-Brain/Fukuyama Congenital Muscular Dystrophy, CMD-MR; Congenital Muscular Dystrophy with Mental Retardation, CMD-no MR Congenital Muscular Dystrophy with no Mental Retardation, CMD-CRB; Congenital Muscular Dystrophy with Cerebellar Involvement, LGMD-MR; Limb Girdle Muscular Dystrophy with Mental Retardation, LGMD-no MR; Limb Girdle Muscular Dystrophy with no Mental Retardation. [b] P; prenatal onset, N; neonatal onset, I; infant onset, Yr; years, m; months. [c] W; walk, S; sit, St; stand, R; run, Prefix N; never. [d-e] Y; yes, N; no. [f] RS; rigid spine, Sc; scoliosis, U; unaffected. [g] CG; congenital glaucoma, RD; retinal detachment, RA; Retinal Atrophy, CC; Congenital cataracts, OA; optic atrophy, My; myopia, Mo; microphthalmia, Pt; ptosis, U; unaffected, Hm; hypermetropia, RDy; retinal dysplasia. [h] UL; Upper limbs, LL; lower limbs, G; generalised, [i] NI; normal intelligence, L; low, [j] Y; yes, N; no, [k] H; Hydrocephalus, CC; cerebellar cysts, BS; brainstem involvement, WM; white matter

abnormality, CHy; cerebellar hypoplasia, Lis; lissencephaly, CDys; cerebellar dysplasia. [1] SE; seizures, CDH; congenital dislocation of hip, RBBB; Right bundle branch block.

Mutation Analysis

Mutation screening of *POMT1*, *POMT2*, *POMGNT1*, *FKTN* and *LARGE* was performed on 92 probands in whom *FKRP* mutations had been previously excluded. Homozygous and compound heterozygous mutations were detected in a total of 31 probands (34 individuals from 31 families). Thirty seven different mutations were identified, 32 of which had not been previously reported. Pathogenic mutations are summarized in Table 8, the comparative pick up between genes is represented in Figure 12 and their locations within each gene are represented In Figure 13.

Without further RNA studies and functional biochemical analysis it is often difficult to determine the pathogenicity of unclassified variants within these genes, this is exacerbated by the abundance of missense variants. For the purposes of this study, nonsense mutations, insertions and deletions, splice site mutations as well as previously reported mutations were classified as pathogenic. Both exonic and intronic sequence alterations were categorized as polymorphisms if they were present on The Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov), the Leiden database (http://www.dmd.nl) or present as an additional change in a patient with two proven pathogenic mutations. Amino acid substitutions were classified as pathogenic if they were detected in conjunction with a clearly pathogenic mutation or if they have been shown to segregate with disease in a large pedigree. In addition, two patients with homozygous missense mutations and one patient with compound heterozygous missense mutations have been included in Table 8 as they are non-conservative amino acid changes that affected an evolutionary conserved amino acid residue (Patient 16, Patient 18 and Patient 27). Patients in whom only a single sequence alteration was detected are summarised in Table 9. We have been unable to determine whether these are rare polymorphisms or pathogenic alterations in patients who harbor a second undetectable mutation. These patients have not been included in the 34% of patients with mutations. Patient 25 has been included in Tables 7, 8 and 10 as well as Figures 12 and 13 despite the absence of a second detectable mutation due to the presence of a nonsense mutation.

A variety of mutation types were identified; 37 missense mutations; 7 nonsense mutations; 9 frameshift mutations; 1 insertion/deletion mutation; 1 deletion and 6 splice site mutations, no mutation hot spots were identified. From a total of 37 mutations, 8 were found to be recurrent within the cohort. The p.Ala200Pro mutation in *POMT1,* previously described as prevalent within the Turkish population (125), was detected in three patients, one of whom was of Greek decent (Patient 8). The *POMGNT1* donor

splice site mutation c.1539+1G>A found to account for the enrichment of MEB within the Finnish population was detected in two patients (259). Three further novel mutations were detected more than once, specifically the p.Tyr666Cys mutation which was found both in the homozygous and heterozygous state in 4 patients. Segregation of this novel missense mutation was studied in a large pedigree and was found to segregate with the disease (Patient 15). Parental samples where studied for 11 probands to ensure that compound heterozygous mutations were in *trans* and that apparent homozygous mutations in the proband were not masking undetected deletions. Where parental DNA was tested (22 families in total) a single paternal mutation was found to occur *de novo* (p.Phe117Ser, *POMT2*). A relatively similar frequency of patients with mutations were detected in *POMT1*, *POMT2*, *POMGNT1* and *FKTN* (Figure 12). In contrast, only a single patient was found to have a pathogenic mutation in *LARGE* although we were unable to confirm a second pathogenic mutation (Patient 25).

Patient	Gene	Exon/ intron	Nucleotide change	Predicted amino acid change	Mutation type	References
1	*POMT1* *POMT1*	20 20	c.2179_2180delTC c.2179_2180delTC	p.Ser727fs p.Ser727fs	Frameshift Frameshift	Novel Novel
2	*POMT1* *POMT1*	20 20	c.2179_2180delTC c.2179_2180delTC	p.Ser727fs p.Ser727fs	Frameshift Frameshift	Novel Novel
3	*POMT1* *POMT1*	7 7	c.598G>C c.598G>C	p.Ala200Pro p.Ala200Pro	Missense Missense	Leiden database Leiden database
4	*POMT1* *POMT1*	18 3	c.1847_1849delGGT c.193G>A	p.Trp616del p.Gly65Arg	Deletion Missense	Novel Leiden database
5	*POMT1* *POMT1*	11 19	c.1081C>T c.2005G>A	p.Gln361X p.Ala669Thr	Nonsense Missense	Novel Novel
6	*POMT1* *POMT1*	6 18	c.517_523delTTCTTCAinsG c.1868G>C	p.Phe173_Asn175delinsAsp p.Arg623Thr	Insertion/deletion Missense	Novel Novel
7	*POMT1* *POMT1*	7 7	c.598G>C c.598G>C	p.Ala200Pro p.Ala200Pro	Missense Missense	Leiden database Leiden database
8	*POMT1* *POMT1*	5 7	c.427G>T c.598G>C	p.Glu143X p.Ala200Pro	Nonsense Missense	Novel Leiden database
9	*POMT2* *POMT2*	21 21	c.2150T>C c.2177G>A	p.Phe717Ser p.Gly726Glu	Missense Missense	Novel (*de novo*) Leiden database
10	*POMT2* *POMT2*	19 19	c.1997A>G c.1997A>G	p.Tyr666Cys p.Tyr666Cys	Missense Missense	Novel Novel
11	*POMT2* *POMT2*	19 11	c.1997A>G c.1238G>C	p.Tyr666Cys p.Arg413Pro	Missense Missense	Novel Novel

Patient	Gene	Exon/ intron	Nucleotide change	Predicted amino acid change	Mutation type	References
12	*POMT2* *POMT2*	20 9	c.2047A>C c.1051delG	p.Thr683Pro p.Ala351fs	Missense Frameshift	Novel Novel
13	*POMT2* *POMT2*	5 19	c.593T>A c.1997A>G	p.Ile198Asn p.Tyr666Cys	Missense Missense	Novel Novel
14	*POMT2* *POMT2*	10 5	c.1117G>T c.593T>A	p.Val373Phe p.Ile198Asn	Missense Missense	Novel Novel
15a, 15b *	*POMT2* *POMT2*	19 19	c.1997A>G c.1997A>G	p.Tyr666Cys p.Tyr666Cys	Missense Missense	Novel Novel
16	*POMT2* *POMT2*	5 21	c.551C>T c.2243G>C	p.Thr184Met p.Trp748Ser	Missense Missense	Novel Novel
17	*POMT2* *POMT2*	9 21	c.1057G>A c.2177G>A	p.Gly353Ser p.Gly726Glu	Missense Missense	Novel [1] Novel [1]
18	*POMGNT1* *POMGNT1*	6 6	c.526A>C c.526A>C	p.Thr176Pro p.Thr176Pro	Missense Missense	Novel Novel
19	*POMGNT1* *POMGNT1*	7 17	c.652+1G>A c.1469G>A	Donor splice site p.Cys490Tyr	Splice site Missense	Novel Leiden database
20 *†	*POMGNT1* *POMGNT1*	20 20	c.1666G>A c.1666G>A	p.Asp556Asn p.Asp556Asn	Missense Missense	Novel [2] Novel [2]
21	*POMGNT1* *POMGNT1*	17 17	c.1539+1G>A c.1539+1G>A	Donor splice site Donor splice site	Splice site Splice site	Leiden database Leiden database
22	*POMGNT1* *POMGNT1*	12 17	c.1100G>A c.1539+1G>A	p.Arg367His Donor splice site	Missense Splice site	Novel Leiden database
23	*POMGNT1* *POMGNT1*	20 20	c.1785+2T>G c.1785+2T>G	Donor splice site Donor splice site	Splice site Splice site	Novel Novel

Patient	Gene	Exon/ intron	Nucleotide change	Predicted amino acid change	Mutation type	References
24	*POMGNT1* *POMGNT1*	17 17	c.1425G>A c.1425G>A	p.Trp475X p.Trp475X	Nonsense Nonsense	Novel Novel
25	*LARGE*	13	c.1548C>G	p.Trp516X	Nonsense	Novel
26	*FKTN* *FKTN*	8 8	c.920G>A c.920G>A	p.Arg307Gln p.Arg307Gln	Missense Missense	Leiden database Leiden database
27	*FKTN* *FKTN*	8 8	c.915G>A c.915G>A	p.Trp305Cys p.Trp305Cys	Missense Missense	Novel Novel
28	*FKTN* *FKTN*	8 8	c.919C>T c.919C>T	p.Arg307X p.Arg307X	Nonsense Nonsense	Novel Novel
29a, 29b	*FKTN* *FKTN*	8 9	c.920G>A c.1167dupA	p.Arg307Gln p.Phe390fs	Missense Frameshift	Novel [3] Leiden database [3]
30	*FKTN* *FKTN*	9 10	c.1167dupA c.1363delG	p.Phe390fs p.Asp455fs	Frameshift Frameshift	Leiden database [3] Novel [3]
31a, 31b	*FKTN* *FKTN*	4 7	c.340G>A c.859delA	p.Ala114Thr p.Thr286fs	Missense Frameshift	Novel Novel

Table 8. A summary of pathogenic mutations detected in this study.

Probands are numbered. Affected siblings are indicated with letters. [*] Family studies carried out to investigate segregation of the variant through the pedigree. [†] Functional characterisation escribed in section 3.2. The following patients have been reported individually; [1] Patients previously described in Mercuri *et al.* 2006. [2] Patients previously described in Clement *et al.* 2007. [3] Patients previously described in Godfrey *et al.* 2006.

Patient	Gene	Exon/intron	Nucleotide change	Predicted amino acid change	Mutation type	References
32	*POMT1*	9	c.905T>G	p.Phe302Cys	missense	Novel
33	*POMT1*	19	c.1922C>T	p.Ala641Val	missense	Novel
34	*POMT1*	20	c.2203C>T	p.Arg735Cys	missense	Novel
35	*POMT1* *POMT1*	20 20	c.2248+5A>G c.2248+5A>G	intronic intronic	intronic intronic	Novel Novel
36	*POMT1*	20	c.2246G>A	synonymous	synonymous	Novel
37,38,39	*POMGNT1*	21	c.1867A>G	p.Met623Val	missense	Novel
39	*LARGE*	4	c.309C>A	synonymous	synonymous	Novel
40	*LARGE*	12	c.1431C>T	synonymous	synonymous	Novel
41	*LARGE*	13	c.1640G>A	p.Arg547His	missense	Novel
42,43	*LARGE*	14	c.1827A>T	synonymous	synonymous	Novel

Table 9. Summary of Unclassified Variants

	WWS	MEB/FCMD	CMD-CRB	CMD-MR	CMD-no MR	LGMD-MR	LGMD-no MR	Total
POMT1	1	1	-	3	-	3	-	**8**
POMT2	-	6	2	-	-	1	-	**9**
POMGNT1	-	6	-	-	-	-	1	**7**
FKTN	1	1	-	-	1	-	3	**6**
LARGE	1	-	-	-	-	-	-	**1**
Mutation detected	3 (60%)	14 (47%)	2 (50%)	3 (20%)	1 (10%)	4 (80%)	4 (20%)	**31 (34%)**
Patient total	**5**	**30**	**4**	**15**	**10**	**5**	**20**	**92** *

Table 10. The phenotypic distribution of patients within the cohort, the frequency of mutations in each of the five glycosyltransferase genes analysed and the comparative mutation frequencies for individual clinical categories.

WWS; Walker-Warburg syndrome, MEB/FCMD; Muscle-Eye-Brain disease/ Fukuyama Congenital Muscular Dystrophy, CMD-CRB; Congenital Muscular Dystrophy with Cerebellar Involvement, CMD-MR; Congenital Muscular Dystrophy with Mental Retardation, CMD-no MR; Congenital Muscular Dystrophy with no Mental Retardation, LGMD-MR; Limb Girdle Muscular Dystrophy with Mental Retardation,

LGMD-no MR; Limb Girdle Muscular Dystrophy with no Mental Retardation. * Includes three patients not assigned a clinical classification due to insufficient clinical information.

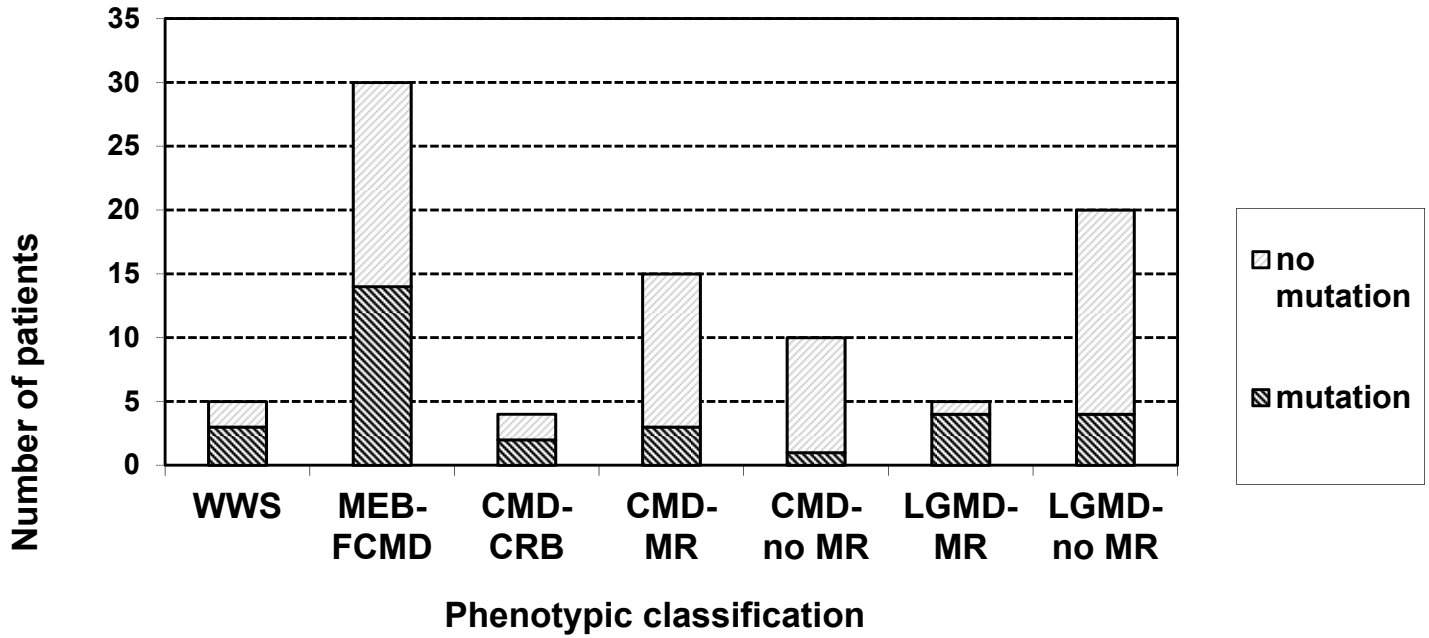


Figure 12. Comparative mutation detection rate for different phenotypic categories.

WWS; Walker-Warburg syndrome, MEB/FCMD; Muscle-Eye-Brain syndrome/ Fukuyama Congenital Muscular Dystrophy, CMD-CRB; Congenital Muscular Dystrophy with Cerebellar Involvement, CMD-MR; Congenital Muscular Dystrophy with Mental Retardation, CMD-no

MR; Congenital Muscular Dystrophy with no Mental Retardation, LGMD-MR; Limb Girdle Muscular Dystrophy with Mental Retardation, LGMD-no MR; Limb Girdle Muscular Dystrophy with no Mental Retardation.

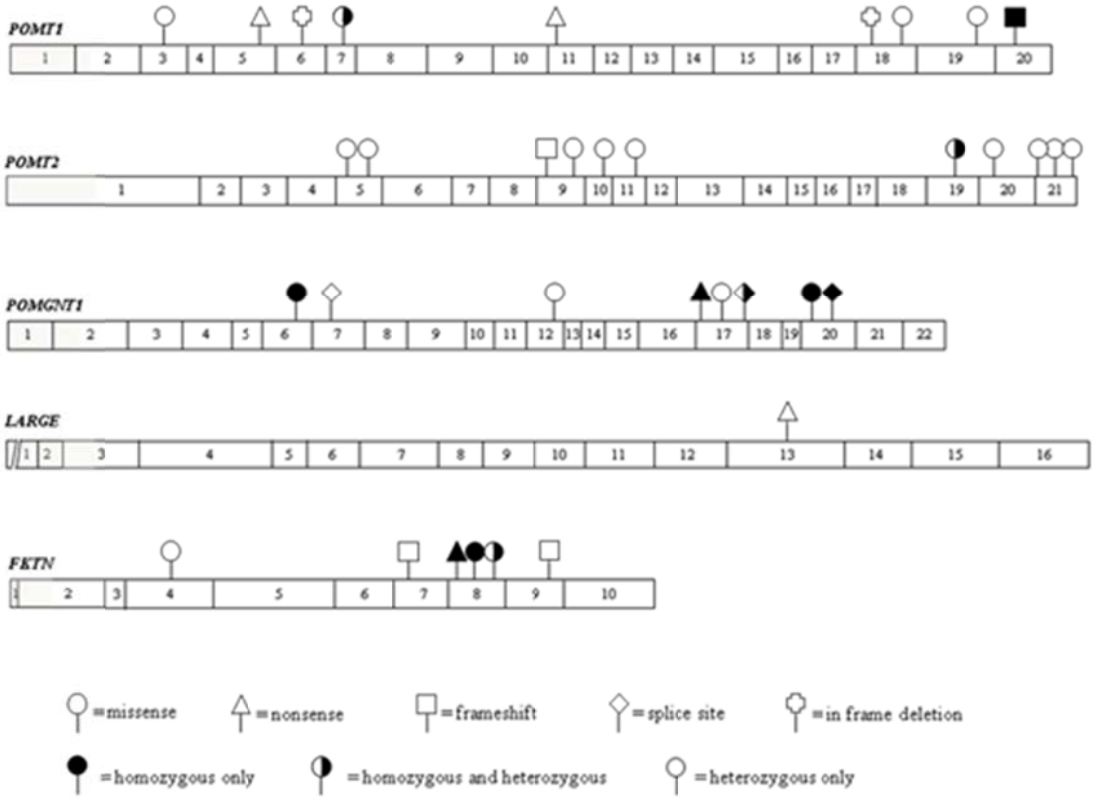


Figure 13. Representation of mutation locations in POMT1, POMT2, POMGNT1, LARGE and FKTN.

White boxes represent individual coding exons, dark grey boxes indicate non-coding exons. All exons are numbered accordingly. The size of the box corresponds to the number of base pairs within each exon. Mutation types and locations are represented by symbols above the appropriate exon; circles represent missense mutations; triangles represent nonsense mutations; squares represent frameshift mutations; diamonds represent splice site mutations; crosses represent in frame deletions. Dark symbols indicate mutations found only in the homozygous state, white symbols indicate mutations found only in the heterozygous state and mixed symbols indicate mutations found in both states.

3.1.4 Discussion

Secondary dystroglycanopathies are a recently defined, common group of muscular dystrophies encompassing an extremely wide spectrum of clinical severity and are caused by mutations in at least 6 genes encoding putative or demonstrated glycosyltransferases. The comparatively small coding region of *FKRP* has facilitated the rapid identification of correlations between genotype and phenotype, allowing the discovery of pathogenic mutations in patients with CMD and LGMD, with and without structural brain involvement. Specific phenotypes attributed to mutations in FKRP include LGMD2I, MDC1C, MEB-like and WWS-like disorders. However, there is no information regarding the frequency of involvement in a large and unbiased population, or on the genotype-phenotype relationships for the remaining 5 glycosyltransferase genes in a large and unbiased population.

In this study we have systematically screened for mutations in *POMT1*, *POMT2*, *POMGNT1*, *FKTN* and *LARGE* in a large cohort of patients in whom we had previously ruled out *FKRP* gene involvement. Mutations were detected in 34% of these patients.

POMT1 mutations

Mutations in *POMT1* have previously been reported in patients with WWS, CMD-MR and LGMD-MR (LGMD-2K). Within our cohort all patients with mutations in *POMT1* had evidence of functional brain involvement, either with no clear associated structural brain abnormalities (as in 3 patients with LGMD-MR (LGMD2K), and in 3 patients with CMD-MR), or more severe conditions with structural brain defects (one patient with WWS, and one individual with a MEB-like phenotype). This suggests that the majority of patients with *POMT1* mutations have either functional or structural central nervous system involvement, including those patients with relatively mild muscle weakness. This is in contrast to our findings reported here for patients with *FKTN* and *POMGNT1* mutations and previously, for *FKRP* mutations (205).

POMT2 mutations

Similarly, mutations in *POMT2* were essentially confined to patients with evidence of brain involvement. Of the 9 patients with pathogenic *POMT2* mutations, 6 were found in individuals with MEB-FCMD. Of the remaining 3 individuals, two have a CMD-cerebellar phenotype, whilst a single LGMD-MR patient (Patient 16) has learning difficulties and remains ambulant at aged 20 having presented at 18 months of age with developmental delay. These findings indicate that as for *POMT1*, the majority, or

indeed all patients with mutations in *POMT2*, do have evidence of central nervous system involvement. In addition, we have identified the mildest phenotype associated with mutations in *POMT2* reported to date in an individual with LGMD-MR.

POMGNT1 mutations

Mutations in *POMGNT1* were also associated with a wider than reported spectrum of clinical severity, which include a relatively mild form of LGMD. However, of the patients in whom *POMGNT1* mutations were identified, 5 had a MEB-like disorder, one a WWS-like disorder with only a single LGMD patient suggesting that *POMGNT1* mutations more frequently give rise to congenital disorders with associated structural brain involvement. Nevertheless, the LGMD patient (Patient 20) has entirely normal intellectual function and onset in the second decade of life which dramatically expands the phenotypes associated with mutations in *POMGNT1.* Whilst initial investigations supported the pathogenicity of the variant found in this family, subsequent functional studies have revealed a more complicated picture which has yet to be fully resolved. This patient is reported in more detail in section 3.2*(260).*

FKTN mutations

Regarding the phenotype initially assigned to mutations in individual genes, our study suggests that the original descriptions of several core phenotypes in this group of disorders are related to the high prevalence of founder mutations within specific populations. Mutations in *FKTN*, typically associated with FCMD in Japan were, for example, found in six patients, none of whom are of Japanese origin. Only 2 of these patients had structural brain involvement, one patient affected by WWS (Patient 28) and one by a MEB-FCMD phenotype (Patient 27). The remaining patients had no structural brain involvement; one case had CMD-no MR (Patient 26) and never acquired the ability to walk but has normal IQ and 5 individuals from 3 families have entirely normal intellect and a mild LGMD phenotype (LGMD2L) (Patients 29, 30, and 31). Interestingly in the latter 2 of these families, febrile illness was accompanied by acute, dramatic deterioration of motor skills which responded to steroid therapy. The muscle biopsy of these patients also contained cellular infiltrate to the extent that polymyositis was considered in the differential diagnosis. These three cases have been reported by us in detail (257). In striking contrast to what has previously been reported in FCMD, none of these 5 patients have evidence of central nervous system involvement. Our findings together with the recent description of individuals with *FKTN* mutations presenting with a predominant cardiomyopathy (32), suggest that *FKTN* mutations do not give rise exclusively to FCMD and that the majority of mutations

outside Japan give rise to milder conditions that are not usually associated with structural brain involvement.

LARGE mutations

Regarding the last gene *LARGE*, we were only able to identify a single pathogenic mutation in a patient with typical WWS phenotype who died in the first few months of life (Patient 25). Unfortunately neither sufficient DNA nor frozen muscle from this patient was available to investigate the presence of a second, as yet undetected, mutation. However, it is highly likely that the nonsense mutation detected is pathogenic, especially since no mutations were found in the remaining glycosyltransferase genes. This compliments the recent report of an intragenic deletion in *LARGE* giving rise to WWS (261).

Mutation frequencies

Concerning the frequency of involvement of individual gene defects in this cohort, mutations in *POMT2* were the most prevalent with 9 cases, followed by *POMT1* with 8 cases, *POMGnT1* with 7 cases, *FKTN* with 6 cases and finally *LARGE* with only a single case. We have previously identified *FKRP* mutations in 79 patients. Approximately 75% of these patients have a LGMD2I phenotype (128, 207, 211, 258). The relative frequency of *FKRP* involvement needs to be considered with caution as it clearly reflects the genetic origin of patients. For example screening of 79 Australian LGMD patients detected only two *FKRP* mutations (229). However, when amalgamating these results, it remains clear that *FKRP* mutations are the most frequently found in this group of conditions. We and others have previously published extensively on the spectrum of these mutations (128, 205, 211, 258, 262-271).

Genotype-phenotype correlations

Regarding the broad correlation between phenotype and genotype for *POMT1*, *POMT2*, *POMGnT1*, *FKTN* and *LARGE*, we detected pathogenic mutations in 3 of 5 patients with WWS syndrome (60%), 14 of 30 patients with a MEB/FCMD phenotype (47%), in 2 of 4 patients with CMD CRB (50%), 3 of 15 patients with CMD-MR (20%), 1 of 10 patients with CMD-no MR (10%), 4 of 5 patients with LGMD-MR (80%), and 4 of 20 patients with LGMD-No MR (20%) (Figure 12 and Table 10).

In most instances there was no apparent difference in the pattern of skeletal muscle weakness or central nervous system involvement in patients with associated structural

brain defects belonging to the severe end of the clinical spectrum. However, 4 LGMD patients with associated MR and microcephaly were all found to have mutations either in *POMT1* or *POMT2*. Conversely a number of patients with considerably more severe muscle weakness compared to brain involvement such as those with CMD-no MR were found to have mutations in *FKTN,* similar to that described in MDC1C (128). A similar pattern has frequently been observed in MDC1C associated with *FKRP* mutations. This suggests that there may be a hierarchical involvement of muscle and brain in the individual conditions, with *POMT1* and *POMT2* being associated with significant central nervous system involvement even in patients with relatively mild weakness who remain ambulant (LGMD2K), while this is not a feature for *FKTN* or *FKRP*. These results suggest that in some individual categories, defects in certain genes are more likely than others, an important consideration when targeting mutation analysis in the dystroglycanopathies.

The results of this study demonstrate that the phenotypic spectrum of disorders associated with mutations in the 6 known glycosyltransferase genes is significantly wider than initially suspected. We have also identified restrictions to the clinical spectrum associated with each gene. We have expanded the clinical spectrum associated with mutations in *POMT1*, *POMT2*, *POMGnT1*, *FKTN* and *LARGE,* further confirming our previous observation for *FKRP* that the severity of the clinical picture may in fact be dependent on the subsequent degree of hypoglycosylation of ADG rather than resulting from a specific gene effect. Finally, this work suggests that more, as yet undefined, genes are likely to be implicated in the pathogenesis of the secondary dystroglycanopathies. The identification of these other genes may provide additional information on the pathway of glycosylation of ADG.

3.2 FURTHER INVESTIGATION OF A MILD POMGNT1 MUTATION

3.2.1 Introduction

Patient 20 in the above cohort was found to have a novel homozygous missense variant in *POMGNT1* (c.1666G>A, p.Asp556Asn). This patient was of particular interest due to the very mild phenotype and prompted further investigation to assess the pathogenicity of the variant.

3.2.2 Clinical History

This patient is one of 5 children born to healthy non-consanguineous Irish parents. She first developed proximal limb muscle weakness at the age of 12 years with difficulty rising from sitting and climbing stairs. Her early motor milestones were normal. Her weakness progressed quite rapidly and at 14 years she was weak proximally more than distally with the neck, hip girdle and shoulder abductors particularly affected. Her Gower's sign was positive. She had hypertrophy of the calves and quadriceps and wasting of the hamstrings and deltoids. She had a lordotic stance and a poor heel strike because of Achilles tendon tightening. Facial expression was normal. Progressive weakness resulted in loss of ambulation at 19 years following a leg fracture. Her general health remains excellent. Her eyesight is very myopic (+6); at the age of 6 years she underwent corrective surgery for a convergent squint. Her intellect is normal and since leaving school she has gone on to university education. Initial investigations included: serum CK consistently elevated between 5000-12000 IU/L and EMG suggestive of a myopathic process.

3.2.3 Methods and Results

Histology and Immunohistochemistry

The muscle biopsy was dystrophic with abnormal variation in fibre size, necrosis, increased endomysial connective tissue and fat, basophilic fibres, some of which were granular and had vacuoles (Figure 14 A). Immunolabeling of β-spectrin, dystrophin and laminin-α2 was normal. Labelling with the IIH6 antibody to the glycosylated epitope of ADG was variable between fibres (Figure 14 C). Some fibres showed a mild reduction while others were very brightly labelled. Similarly labelling with the core antibody was weaker on some small fibres (Figure 14 D). A proportion of these fibres were also weaker with the anti BDG antibody and may correspond to basophilic fibres (Fig 14 B).

Further analysis of the biopsy showed normal Western blot analysis of dystrophin, sarcoglycans, laminin-α2, caveolin, emerin, calpain 3, dysferlin and telethonin (carried out in the diagnostic Limb Girdle Muscular Dystrophies NSCAG centre in Newcastle, UK, courtesy of Prof K. Bushby).

Single Section Western blotting and overlay assays

Western blot and overlay analysis of skeletal muscle was performed by Dr Silvia Torelli. Western blot showed an expression of ADG similar to the control using an antibody which recognises a glycosylated epitope (IIH6; Fig 15 A). BDG expression was normal and demonstrates equal protein loading. The laminin overlay assay showed that ADG's ability to bind laminin was similar to control (Fig 15 B).

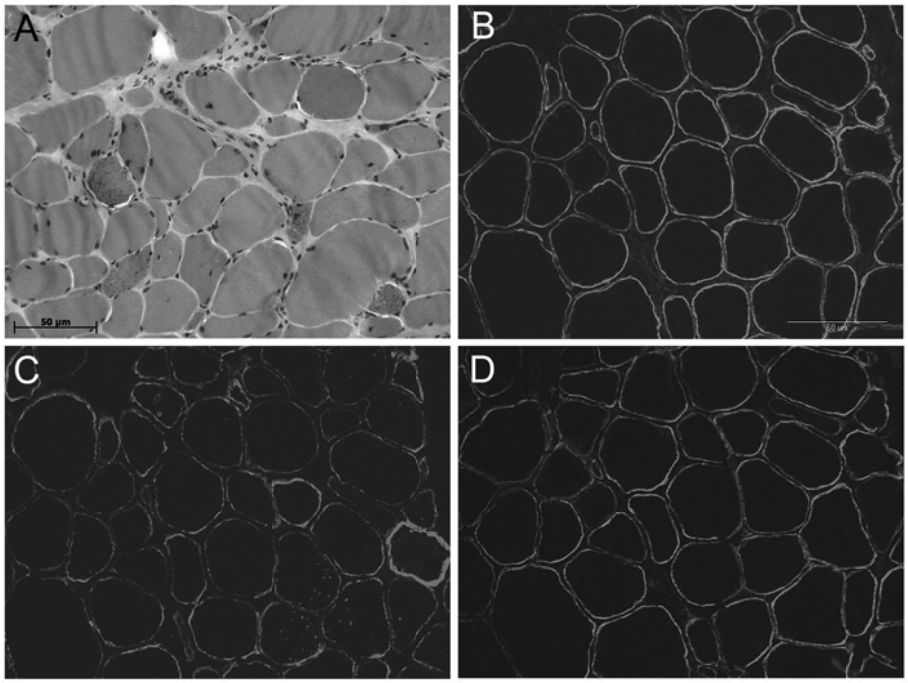


Figure 14. Skeletal Muscle Biopsy from patient 20.

A. Hematoxylin-eosin staining showing dystrophic appearance and granular fibres with vacuoles.
B. Immunolabelling for BDG with some fibres staining weaker than others. This may correspond to basophilic fibres.
C. Immunolabelling for the glycosylated epitope of ADG with IIH6 antibody showed intrafibre variability.
D. Immunolabelling for ADG using core antibody appeared weaker on some small fibres.

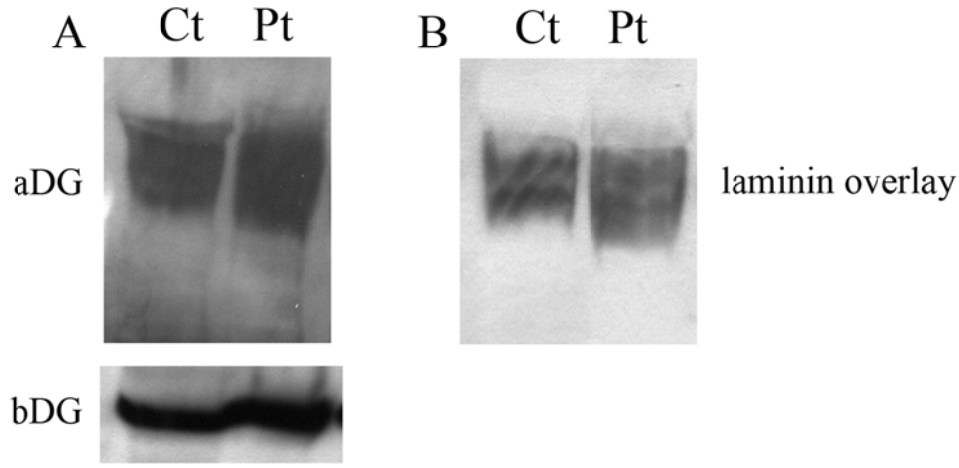


Figure 15. Single-section skeletal muscle analysis.

A, Western Blot analysis using the IIH6 antibody (ADG) shows that expression and molecular weight of patient muscle were similar to those of control muscle. BDG expression was similar to control and demonstrates equal loading between samples.
B, Laminin overlay assay. The patients ADG shows the same ability to bind laminin compared with control muscle.

Ct; control, Pt; patient 20.

Mutation Analysis

A homozygous single nucleotide change c.1666G>A, never described before, was detected in exon 20 of *POMGNT1*. This change is predicted to result in the substitution of a conserved aspartic acid at amino acid 556 to asparagine (p.Asp556Asn) in the POMGNT1 protein. No other sequence variations were detected in the other known secondary dystroglycanopathy genes (*FKRP, POMT1, POMT2, FKTN or LARGE)* and linkage to the MDC1B locus on 1q42 was excluded (28). Both parents were found to be carriers of the mutation and none of the four unaffected siblings tested were homozygous for this change. This alteration was not detected in over 100 disease controls.

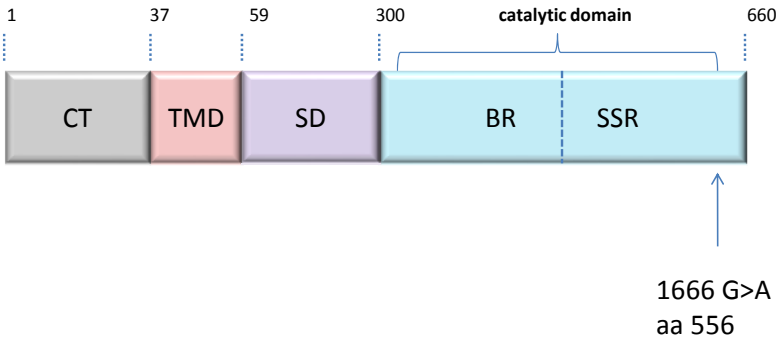


Figure 16. Diagram showing pictorial representation of POMGNT1 and the position of the amino acid change.

POMGNT1 is comprised of 660 amino acids and contains a CT; cytoplasmic tail, TMD; transmembrane domain, SD; stem domain, and a catalytic domain comprising a binding region (BR) and substrate specific region (SSR). The mutation amino acid change in patient 20 is at position 556 and substitutes an aspartic acid for asparagine.

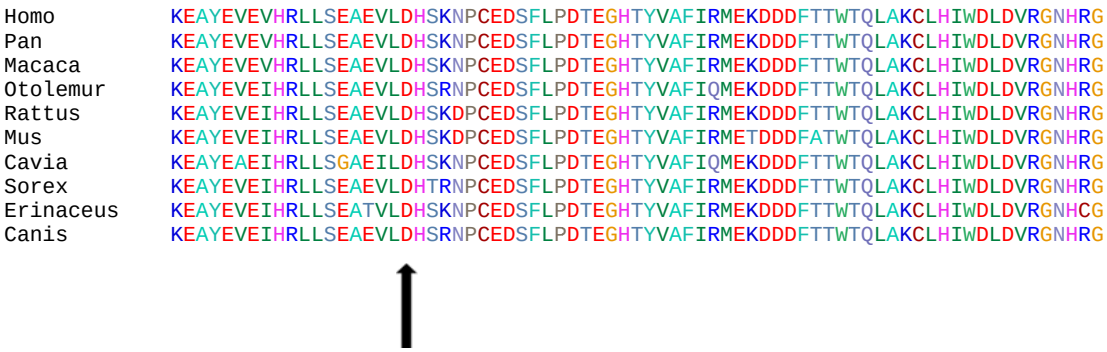


Figure 17. Homology alignment depicting a region of the catalytic domain of POMGNT1.

Arrow marks position 556 which is conserved in the ten vertebrate species shown.

Kinetic analysis of POMGNT1 enzyme activity

In order to demonstrate the pathogenicity of this sequence variant, we arranged for kinetic analysis of POMGNT1 to be carried out by Professor Harry Schachter's group in the department of Structural Biology and Biochemistry in Toronto, Canada.

Kinetic analysis of POMGNT1 activity at 4 different concentrations of UDP-GlcNAc (Figure 18 A) shows apparent Km values of 2.2 and 6.1 mM for control and patient fibroblasts respectively. The respective apparent Vmax values are 3.4 and 2.5 nmoles/hr/mg protein; this difference is not statistically significant. It is not possible to obtain accurate Km and Vmax values for Man-α-1-O-benzyl with normal POMGNT1 because these parameters are both so large that a plot of enzyme rate versus acceptor concentration shows a straight line through the origin, up to the maximum soluble concentration of 60 mM Man-α-1-O-benzyl (272). However, a similar analysis of patient fibroblasts (Figure 18 B) shows a qualitative change from a straight line to a classical hyperbolic Michaelis-Menten plot (apparent Km and Vmax values of 9 mM and 0.1 nmoles/hr/mg protein respectively).

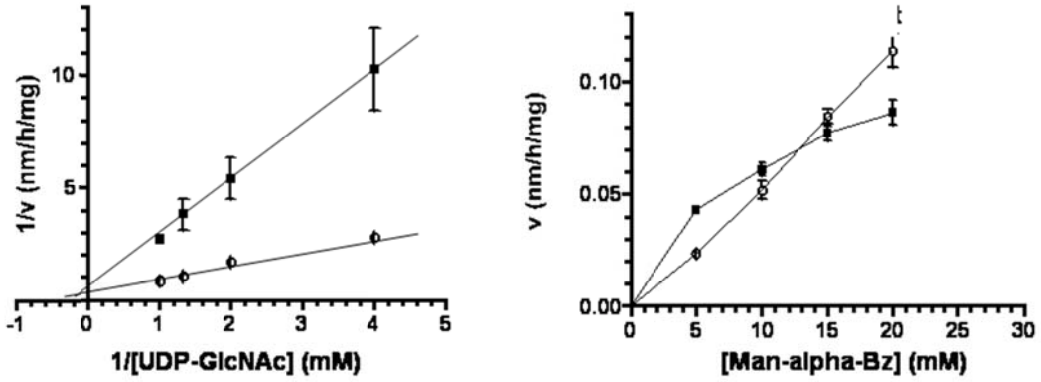


Figure 18. Kinetic Analysis of POMGNT1 activity in patient 20.

Figure 18 A. A reciprocal plot of enzyme rate (v, nmoles/hr/mg protein) versus UDP-GlcNAc concentration (0.25, 0.5, 0.75, 1.0 mM) at 62.5 mM Man-α-1-O-benzyl for control (circle) and patient (square) fibroblasts. The plots are linear showing classical Michaelis-Menten behaviour. The apparent Km and Vmax values are, respectively, 2.2 and 6.1 mM (highly significant difference) and 3.4 and 2.5 nmoles/hr/mg (difference not statistically significant) for control and patient fibroblasts. Standard deviation bars are shown.

Figure 18 B. A plot of enzyme rate (v, nmoles/hr/mg protein) versus Man-α-1-O-benzyl concentration (5, 10, 15, 20 mM) at 0.5 mM UDP-GlcNAc for control and patient fibroblasts. The plot for the control fibroblasts is linear as previously described14 indicating relatively large Km and Vmax values. The plot for patient fibroblasts is hyperbolic. The reciprocal plot (not shown) is linear yielding apparent Km and Vmax values of 9 mM and 0.1 nmoles/hr/mg respectively. The slopes of the two reciprocal plots are significantly different at the 95% confidence level.

3.2.4 Discussion

To date, the identification of *POMGNT1* mutations has been restricted to patients with CMD and brain abnormalities. The majority of these patients have a phenotype consistent with MEB, although some more severe cases resembling WWS have also been reported(129, 254).

One of the unanswered questions regarding the genotype-phenotype correlation for *POMGNT1* mutations relates to whether it can cause mild as well as severe phenotypes as seen in other secondary dystroglycanopathy genes, most notably *FKRP* (192) (226) but more recently also *FKTN* (257).

The p.Asp556Asn mutation reported here is predicted to be located in the substrate specific region of the catalytic domain. Several lines of evidence indicate that this alteration is pathogenic. Firstly the patient's skeletal muscle biopsy is consistent with a muscular dystrophy and suggestive of a dystroglycanopathy. No mutations were detected in the five other dystroglycanopathy genes screened. The reduction of ADG labelling was subtle on immunocytochemistry and no significant reduction in molecular weight was observed on Western blot. This highlights the difficulty in detecting mild ADG abnormalities and has been previously observed with mild *FKRP* mutations in LGMD2I(256). Secondly the mutation segregated with the disease in this relatively large family. Thirdly, the mutation causes a reversal of amino acid polarity in the substrate specific domain of POMGNT1 which may be expected to have functional consequences. In addition, the detailed enzymatic studies offer support for this theory. POMGNT1 activity is observed but differs in its kinetics from that of control samples. The apparent Km for UDP-GlcNAc is significantly higher and the apparent Vmax for Man-α-1-O-benzyl is significantly lower than in the control. Application of the POMGNT1 assay conditions that were previously used to analyze MEB patients (1 mM UDP-GlcNAc, 62.5 mM Man-α-1-O-benzyl) (219) to the LGMD patient in this study, yields a POMGNT1 rate that is significantly higher than the values found in MEB patients. Reliable diagnosis of patients such as the one in this study therefore requires determination of kinetic parameters.

It is interesting to note that this mutation had no effect on the subcellular localisation or expression of a recombinant form of *POMGNT1* when overexpressed in C2C12 myotubes (data not shown, performed by Martin Brockington, Caroline Godfrey and Silvia Torelli). However, the p.Asp556Asn mutation introduces a potential *N*-

glycosylation site (Asn-X-Ser) into the protein which is predicted to have no other such sites. The presence of an *N*-glycan at this position may cause defective enzyme folding that results in an active but inefficient enzyme.

This report significantly expands the spectrum of disorders associated with *POMGNT1* mutations and this patient is the mildest POMGNT1 deficient patient described. The finding of normal POMGNT1 activity on conventional enzymatic assay but the identification of altered kinetic properties of the mutant enzyme highlights the importance of careful interpretation of functional data especially when studying atypical clinical presentations(260).

Since the publication of this data, further information has come to light clouding the issue of mutation pathogenicity in this family.

Firstly, although the mutation was excluded from being a common polymorphism on the basis that it was not found in 100 disease controls or present in the dbSNP, Caroline Godfrey later found the variant in 1 of 150 European disease control chromosomes. Secondly, following publication of the data, we were informed that the same *POMGNT1* substitution had been observed in a French kindred. In this family a patient with MEB and her unaffected sibling both harboured the homozygous change. It was also reported in 8 out of 218 healthy French control chromosomes (Celin Bouchet, personal communication). This analysis was going to be repeated by our colleagues in France but we have never been informed about the outcome of the repeated studies. Although this evidence throws doubt on the pathogenicity of the change identified, we cannot ignore the fact that our patient showed an altered POMGNT1 kinetic profile. It may be that this is a secondary effect to a mutation in another glycosyltransferase gene or that other moderating factors are implicated in the complex kinetic profile of this patient. Although we would have liked to have investigated this situation in more depth, to date, further studies have not been undertaken as we are not yet in possession of fibroblasts from the French unaffected sibling to assess POMGNT1 function in this family.

CHAPTER 4: REFINING THE SPECTRUM OF BRAIN ABNORMALITIES IN THE DYSTROGLYCANOPATHIES

4.1 INTRODUCTION

Brain involvement is a frequent but not consistent feature in patients with dystroglycanopathies. At the most severe end of the spectrum, in patients with WWS and MEB, patients are affected by cobblestone (type 2) lissencephaly with gross structural changes affecting cerebral cortex, brainstem and cerebellum and invariably associated with mental retardation. Conversely, patients with milder dystroglycanopathy phenotypes such as LGMD2I have no detectable brain abnormality on MRI imaging and normal cognitive functioning. As with other phenotypic findings in this group of conditions, a complete spectrum exists in between the 2 extremes. Here we explore the various brain abnormalities seen in a large cohort of dystroglycanopathy patients with proven mutations in either *POMT1, POMT2, POMGNT1, FKTN* and *LARGE* genes seeking to comprehensively document the findings and outline any evident genotype phenotype relationship.

4.1.1 Central Nervous System Development

The central nervous system (CNS) is derived from the dorsal epiblast of the vertebrate embryo and is induced by a combination of signals resulting in the formation of a neural tube. Anterior posterior and dorsoventral patterning occurs resulting in the formation of the prosencephalon (forebrain) which then subdivides into diencephalon and telencephalon, the mesencephalon (midbrain) and the rhombencephalon (hindbrain) which further divides in to the metencephalon (forming pons and cerebellum) and myelencephalon (medulla oblongata)(273) Correct patterning is dependent on the interaction of numerous genes including PAX2, PAX6, EN1, EN2, Wnt and sonic hedgehog.

4.1.1.1 Normal Cortical Development

The development of the human cerebral cortex can be divided into 3 overlapping stages. During the first stage, cells proliferate into glial cells in the ventricular and subventricular zones lining the cerebral cavity, deep in the forebrain. Postmitotic cortical neurones then migrate towards the pial basement membranes on radial glia that cover the neuroepithelium, with endfeet attached to the pial surface. Migrating neurones split the preplate into the subplate (SP) and marginal zone (MZ), stopping in the cortical plate (CP). Each successive generation of neurones passes the previous, forming an inside out pattern within the CP. Finally, cortical organisation occurs,

regulated by reelin released by Cajal-Retzius cells in the marginal zone, resulting in a 6 layered cortical plate (Figure 19). This is a dynamic process, in humans the proliferation stage occurs between 5-6 and 16-20 weeks, the migration stage between 6-7 and 20-14 weeks and organisation begins at week 16 and continues into postnatal life.(171, 274, 275). When migration is complete, the six layered cortex comprises different types of neurones that form discrete connections within the CNS and perform different functions (276).

4.1.1.2 Abnormalities of Cortical Development

A classification system for malformations of cortical development has been devised and is based on the three general steps of cortical development. Abnormalities that primarily affect proliferation or apoptosis result in abnormal glia and neuronal differentiation causing abnormal cell size and morphology. This group would include disorders such as megalencephalies and some neoplastic disorders such as gangliocytomas. Disorders affecting neuronal migration result in abnormal neuronal positioning and include conditions such as periventriuclar nodular heterotopia, lissencephaly, and cobblestone complex. Disorders affecting later cortical development and organisation are likely to result in abnormalities restricted to the cortex, such as polymicrogyria and schizencephaly (277) (275).

<u>Neuronal Migration Disorders and Lissencephaly</u>

Many subtypes of neuronal migration disorders have been reported and can broadly be classified according the stage of migration at which the defect occurs. In some cases, a proportion of migrating neurones may not manage to leave the ventricular zone (VZ). In humans this results in periventricular heterotopia (PH) and may be caused by mutations in *filamin 1 (FLN1)*. Abnormalities that occur in neurones after they exit the VZ may result in the appearance of a smooth cerebral cortex known as type 1 lissencephaly. In such cases neurones leave the VZ but then arrest, resulting in a 4 layered cortex that bears little resemblance to the 6 layered cortex seen in the normal human brain. Mutations in *LIS1* and *DCX* may result in a type 1 lissencephaly phenotype. In other disorders, the defect may be later, with ineffective penetration of migrating neurones through the subplate, prior to cortical organisation. In some cases (as in the reeler mouse, null for reelin) this leads to inversion of the normal inside out pattern of cortical migration and an excess of neurones in the MZ. In patients with type 2 lissencephaly, the defect is with the expansion of the developing cerebral wall, which

results in abnormal neuronal migration. Type 2 lissencephaly, as seen in dystroglycanopathy cases, has the appearance of a smooth brain on brain imaging but, at the histopathological level, breaches in the pial surface lead to over migration of neurones onto the subarachnoid tissue, giving the appearance of verrucose dysplasia and characteristic cobblestone appearance. Impaired anchoring of radial glia processes may be the primary cause for the migration defect, similar to the abnormalities seen in mice null for β1 integrins in the CNS or those with mutations in the laminin γ1 chain (Figure 19) (171, 278-280).

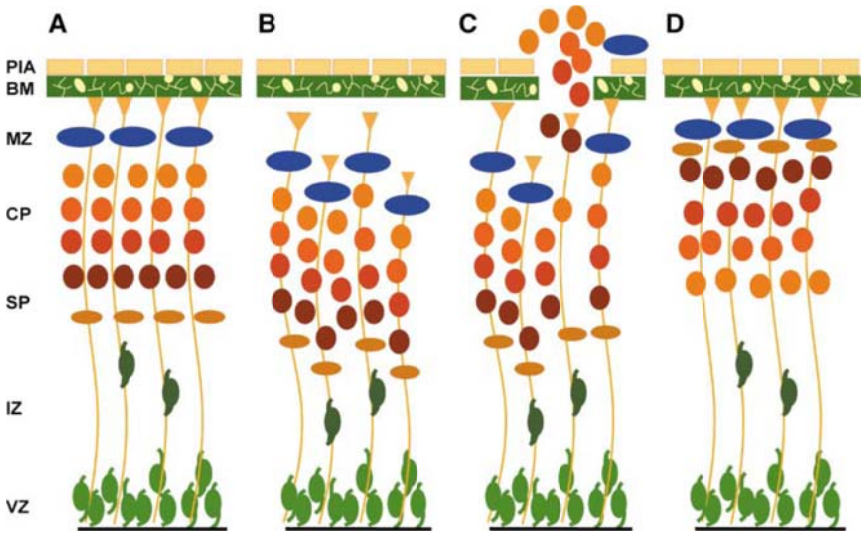


Figure 19. Abnormalities in Cortical Development.

(A) During normal cortical development, neuroblasts (light green) proliferate in the ventricular zone (VZ), giving rise to postmitotic neurons (dark green) that migrate toward the marginal zone (MZ) along radial glia processes (orange). Postmitotic neurons migrate past subplate cells (SP) and then detach from the radial glia once they have reached their proper positions within the cortical plate (CP). Position is regulated by their time of birth and by reelin released by Cajal-Retzius cells (blue) located in the MZ. First-born neurons (dark red) stop close to SP cells, while older neurons (progressively lighter shades of red) must migrate past them to form an inside-out layering scheme. Three examples of migration disorders are shown. (B) Radial glia processes fail to attach to the basement membrane (BM) and do not extend into the MZ. The inside-out pattern is preserved, but Cajal-Retzius cells are located at varying distances from the BM. Neuronal layers are not aligned, and the CP has a wavy appearance. This is known to occur in mouse brains deficient in β1 integrins. (C) Focal disruptions of the BM allow Cajal-Retzius cells and neurons to migrate into the adjacent subarachnoid space. These heterotopia are seen in mice deficient in β1 integrins or with mutations in the laminin γ1 chain. (D) Neurons do not respond properly to a migration stop signal. Signaling between neurons and Cajal-Retzius cells is necessary for migration arrest and neuronal positioning in the CP. In mice mutant for reelin, neurons do not migrate past SP cells, and the inside-out pattern of neuronal layering is roughly inverted.

Adapted from Montanaro *et al.* with permission(171).

Polymicrogyria

Polymicrogyria (PMG) is a relatively common malformation of cortical development, characterised by multiple small gyri and shallow sulci giving the cortical surface and cortical white matter junction a convoluted appearance. It is an aetiologically heterogeneous condition although several distinct PMG syndromes are now recognised (274). Studies in excitotoxic animal models suggest that PMG results from a developmental disorder or insult that affects the neurones towards the end of the migration or early cortical organisation phase. Other studies show that in humans, cytotoxic insult or hypoperfusion between weeks 16 and 24 may result in a similar picture. Piao *et al.* examined the expression pattern of *GPR56*, a gene known to be mutated in autosomal recessive bilateral frontoparietal PMG, and proposed that the insult may happen earlier, during neuronal proliferation and migration. The weight of evidence suggests that PMG is the end point of a number of different aetiological processes (281-283). PMG may be focal or diffuse, unilateral or bilateral and can occur in isolation or in conjunction with other features. PMG is a frequently observed feature in dystroglycanopathy patients (284).

Pathological features of PMG

Microscopically two types of PMG are recognised; 4 layered and unlayered, which often coexisit in contiguous areas suggesting that they may not be distinct malformations. Four layered PMG is comprised of a molecular layer and 2 layers of neurones separated by an intermediate cell-sparse layer containing a few cells and myelinated fibres. Unlayered PMG has a continuous molecular layer and a layer of neurones without laminar organisation. Unlayered PMG is likely to reflect an early disruption of normal neuronal migration with subsequent disordered cortical organisation. Four layered PMG probably results from later disruption in neuronal migration or cortical organisation(277) (274).

Imaging Features of PMG

The diagnosis of PMG can be made by observing a combination of features on thin slice MRI imaging; abnormal gyral pattern, irregularity of the cortical white matter junction and in some cases increased cortical thickness. Cortical thickness may be altered by myelination and hence increased thickness is not seen on early scans. As the brain matures and undergoes myelination of subcortical and intracortical fibres, the pattern evolves and increased cortical thickness may then be detected on T2 weighted images (Figure 20)(285).

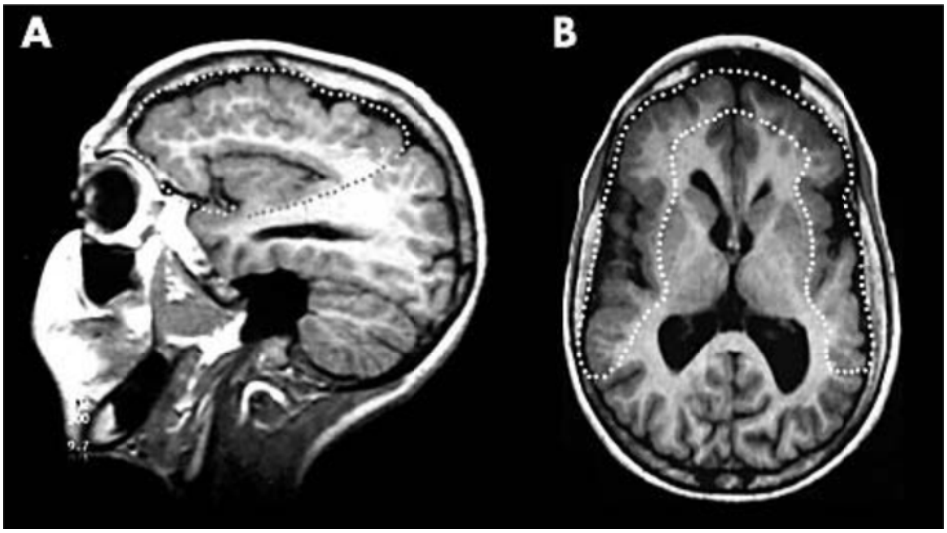


Figure 20. Bilateral frontal polymicrogyria (BFPP) in a patient with GPR56 mutation.

Sagittal (A) and axial (B) T1 weighted magnetic resonance images displaying irregular, bumpy appearance of the hemispheric contour, shallow sulci, and nodular appearance of the cortex. The dotted contour shows the extent of the abnormal cortex, with predominant frontal involvement.

Reproduced from Jansen *et al*. 2004, with permission (274).

4.1.1.3 Developmental Abnormalities of Mid and Hindbrain

A system has recently been proposed by Barkovich *et al.* that attempts to classify the disorders of the midbrain and hindbrain(286). They are a huge, complex and heterogenous group of disorders and comprise isolated malformations and those that are seen in conjunction with additional brain or other systemic abnormalities. The system proposed is based largely on embryology and genetics and in that regard is similar to the system proposed for abnormalities of cortical development. Of the 4 major classification groups, group 2 comprises 'malformations associated with generalized developmental disorders that significantly affect the brainstem and cerebellum'. This group is further subdivided and includes 'malformation of neuronal migration that prominently affect the brainstem and cerebellum' which is again divided and includes 'malformations with basement membrane and neuronal migration defects' which in turn includes the dystroglycanopathies.

A number of different abnormalities have been reported in the dystroglycanopathies with varying degrees of severity. These include thinning of the brainstem, midbrain kink, enlarged tectum and cerebellar dysplasia, hypoplasia and cysts. The cerebellar abnormalities may be caused by disturbances in the external granule layer (287). The small pons and midline cleft may result from hypoplasia of the decussating fibres of the middle cerebellar peduncles or from impaired tangential migration of pontine nuclear neurones as shown in mouse models of *LARGE* mutations (288). As in the cerebral abnormalities, the midbrain-hindbrain disorder appears to be the result of both abnormal neuronal migration and abnormal formation of white matter tracts.

4.1.1.4 Dystroglycan in the Brain

Alpha Dystroglycan (ADG) is known to be expressed in the normal brain, where its molecular mass is 120kDa, suggesting a different glycosylation profile to that observed in muscle where its molecular mass is 200kDa. In mice glycosylated ADG has been shown to be particularly prevalent in two CNS locations; firstly in the astrocyte foot processes that form the glia limitans at the pial surface of the brain and spinal cord and secondly in foot processes surrounding cerebral microvessels (289). It is also found in the hippocampus and cerebellar cortex, where it is thought to have a structural role in synapses. The pathological changes observed in dystroglycanopathy patients are thought largely to be a result of defective basement membrane and the consequential disruption of neuronal migration. However, in the dystroglycanopathies, disrupted neurological interactions in migrating neurones may also play a part as antibodies

directed against glycosylated ADG may affect granule neurone migration (290). Interestingly dystroglycan, *FKTN* and *POMGNT1* are all expressed in migrating cerebellar neurones and also in glial scaffolds used during radial migration. Abnormal glycosylation of ADG on the glial scaffolds may in turn affect interaction with ADG ligands during migration, suggesting an additional way in which neuronal migration may be compromised (287). Glycosylated ADG is also found in several ocular locations; extraocular muscle membranes, retina inner limiting membranes, foot processes surrounding microvessels and puncta in the outer plexiform layer (163).

Dg^{null} mouse

Mice null for dystroglycan do not develop beyond embryonic day 6.5 due to lack of formation of Reicharts basement membrane (161).

Dg^{-CNS} mouse

Studies by Moore *et al*. on mice with a dystroglycan-null brain phenotype (GFAP-Cre/DG null) revealed dramatic abnormalities that mirrored pathological findings in the brains of patients with cobblestone complex, associated with the more severe dystroglycanopathy phenotypes. Specifically they found that brain selective dystroglycan deletion caused microcephaly, aberrant cerebral cortical layering, fusion of the cerebral hemispheres and cerebellar folia and abnormal migration of granule cells. The dystroglycan null brain also lost high affinity binding to laminin and showed discontinuity in the pial surface of the basal lamina, which they hypothesised contributed to the neuronal migration abnormality observed (146).

Myd^{LARGE} mouse

Further supportive evidence of the central involvement of ADG in the brain abnormalities seen in dystroglycanopathy patients came from observations of the *myd*^{LARGE} mouse. The normal expression pattern of glycosylated ADG is not observed in brain tissue from these mice and a pathological phenotype is found that, whilst less severe, resembles that seen *Dg*$^{-CNS.}$ Abnormalities reported include abnormal neuronal migration in the cerebral cortex, cerebellum and hippocampus and disruption of the basal lamina. Analysis of the brainstem has revealed ectopic neurones of the pontine nuclei, thought to occur as a result of defective tangential neuronal migration (288). Interestingly, neuronal layering and radial glia in the retina appear to be normal in the *myd* mouse(163).

Pomgnt1^{null} mouse

Hu *et al.* studied the neuronal migration abnormalities observed in *Pomgnt1*^{null} mice and demonstrated that the overmigration was secondary to abnormalities in the pial basement membrane and disappearance of the glia limitans. They suggested that the disappearance of the pia mater in this mouse may be as consequence of weakened cell-ECM interactions and subsequent reduced ability to bear mechanical stress secondary to hypoglycosylation of ADG(291).

Fktn and *Pomt1* mice

Mice homozygously deleted for *Fktn* or *Pomt1* are embryonically lethal due to defects in the basement membrane (292, 293). Chimeric *Fktn* deficient knockdown mice are viable. These mice have brain abnormalities including breaches of neurones through the glia limitans into the subarachnoid space. Studies have suggested that the these breaches occur as a result of abnormalities in the basement membrane rather than the migrating neurones, similar to the conclusions for the *Pomgnt1*^{null} mouse(294).

4.1.1.5 Dystroglycanopathy Brain Phenotypes

Not all patients with a dystroglycanopathy have brain abnormalities. Severe structural and functional brain involvement is however a constant feature of WWS, MEB and FCMD. Central to these disorders is the finding of 'cobblestone cortex' but they also display in varying severity, ventricular dilatation, white matter changes which may be transient and abnormalities of the brainstem and cerebellum. These features are clearly evident on MRI brain scan.

Walker –Warburg Syndrome.

In 1984, Towfighi *et al*. reported the neuropathology of several cases with Cerebro-ocular dysplasia- muscular dystrophy, the clinical descriptions of which are consistent with WWS. In these cases, the cerebral malformations included lissencephaly, with varying degrees of polymicrogyria, hypomyelination of cerebral white matter, diffuse leptomenigeal and marginal gliomesodermal proliferation, cerebellar polymicrogyria, and hypoplasia or absence of pyramidal tracts. Gross structural abnormalities included hydrocephalus and partial fusion of the frontal lobes(240).

Several papers have reported the variety of MRI brain changes in patients with WWS. The gross appearance is that of widespread aygria with scattered areas of macrogyria

and/or polymicrogyria, giving rise to type 2 lissencephaly, which represents the most severe end of the cobblestone complex spectrum. The leptomeninges are thickened and may obliterate the interhemispheric fissure, ‘fusing’ the cortex(241). Brainstem abnormalities include thin brainstem, kink at the junction of the midbrain and pons, enlarged rounded tectum with fusion of superior and inferior colliculi. The pons is hypoplastic with a ventral midline cleft sometimes apparent on axial images. The cerebellum is generally small and highly dysplastic(295). The vermis, especially the posterior vermis is always hypoplastic, often associated with enlargement of the fourth ventricle and Dandy Walker Malformation. Posterior cephaloceles are also widely reported and are seen in 25-50% of cases (241). Ventricular dilatation and hydrocephalus is almost universally seen (Figure 21).

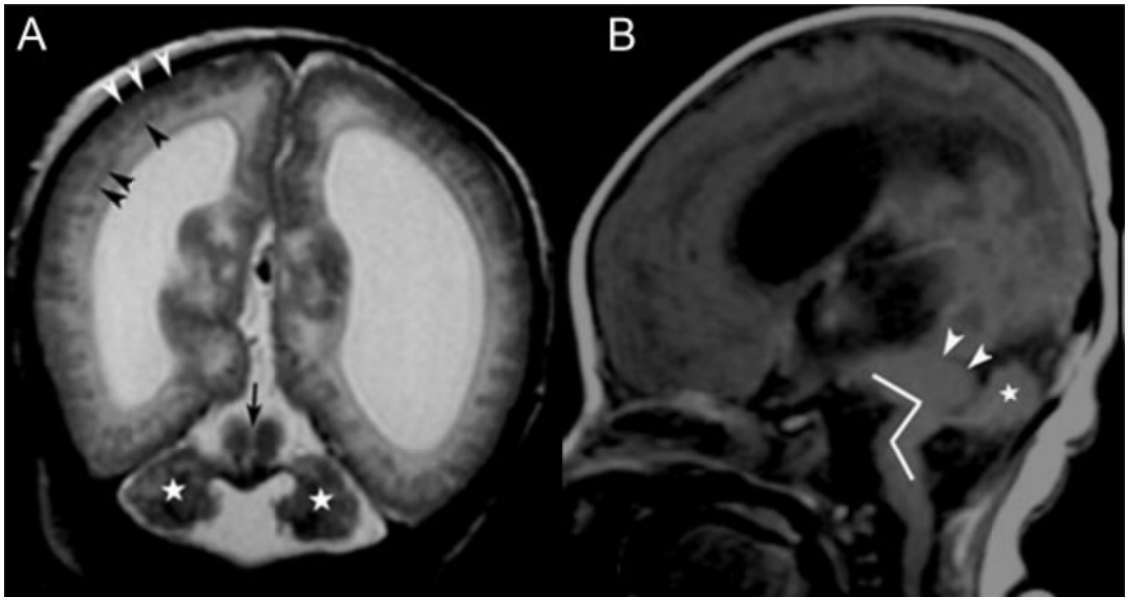


Figure 21. Cobblestone Complex in WWS.

(A) Coronal T2-weighted image showing typical magnetic resonance appearance of cobblestone complex with irregular inner layer of ectopic neurons (black arrowheads) and overmigrated neurons at the surface of the cortex (white arrowheads) and between both fibroglial bundles crossing the white matter radially, hydrocephalus, and profound hypomyelination. Midbrain and hindbrain (white stars on cerebellar hemispheres) are severely hypoplastic with near total absence of the vermis and pontine midline cleft (black arrow).
(B) Sagittal T1-weighted image showing the characteristic dysplastic large tectum (arrowheads), deformity of the mid-hindbrain junction (pontomedullary “kink,” white line), and near total absence of the vermis with very hypoplastic cerebellar hemisphere (white star).

Reproduced from Jissendi-Tchofo *et al.* 2009 Neurology (296) with permission.

Muscle-Eye-Brain Disease.

The first reported neuropathological studies of patients with MEB revealed cortical abnormalities including occipital agyria and disorganised cortical lamination and a nodular surface consistent with cobblestone cortex (Figure 22) (297). In addition, the surface of the cerebellum was devoid of normal folia, the vermis was hypoplastic and the pons was flattened. The microscopic cytoarchitectural disorganisation was similar to that seen in the cerebral cortex of both WWS and FCMD.

MRI brain changes in MEB include cobblestone cortex, white matter changes, thinning and sometimes kinking of the brainstem with small ventral pontine cleft. Cerebellar abnormalities include dysplasia, hypoplasia and cysts (Figure 23) (298).

Fukuyama Congenital Muscular Dystrophy.

The findings in FCMD are similar to those in MEB and WWS. Cobblestone complex, pachygyria and polymicrogyria are features and dilated ventricles and white matter abnormality are also seen. Midbrain hypoplasia is present in some and cerebellar polymicrogyria and cysts are also a frequent observation (23 of 25 individuals in one study) (Figure 24) (299).

Figure 22. Pathological appearance of brain in a patient with MEB.

An occipital agyric area (left) contrasts with the abnormal and patterned gyri 'cobblestone cortex' of the rest of the brain surface.

Reproduced from Haltia *et al.* (297) with permission.

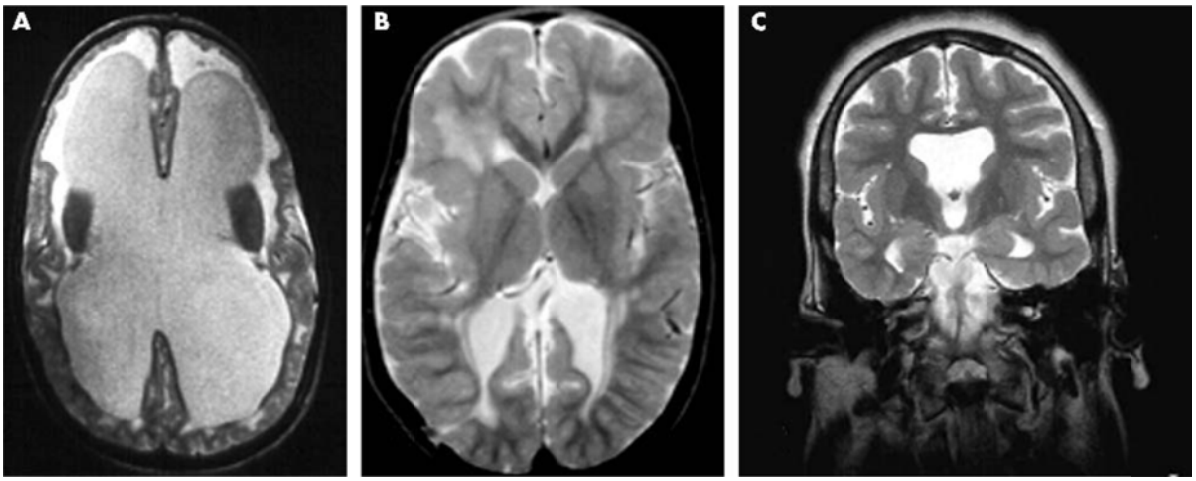


Figure 23. T2 weighted images of three patients with muscle-eye-brain disease (MEB) homozygous for the Finnish founder mutation.

(A) In an axial image of a clinically severe case, the lateral ventricles are extremely large and the interventricular septum is missing. There is hyperintensity and volume loss of the entire white matter. The abnormal cortex is clearly visible, especially in the frontal lobes.
(B) In a clinically intermediate patient with a ventriculoperitoneal shunt, an axial image shows white matter hyperintensity around the ventricles in the frontal and peritrigonal area. The rest of the white matter shows normal intensity. Note the cobblestone pattern of the frontal cortex.
(C) In a clinically mild case, a coronal image shows mild dilatation of the ventricles with absent interventricular septum. The white matter intensity is normal. The abnormal cortex is again seen in the frontal lobes.

Reproduced from Diesen *et al.* 2004 (259) with permission.

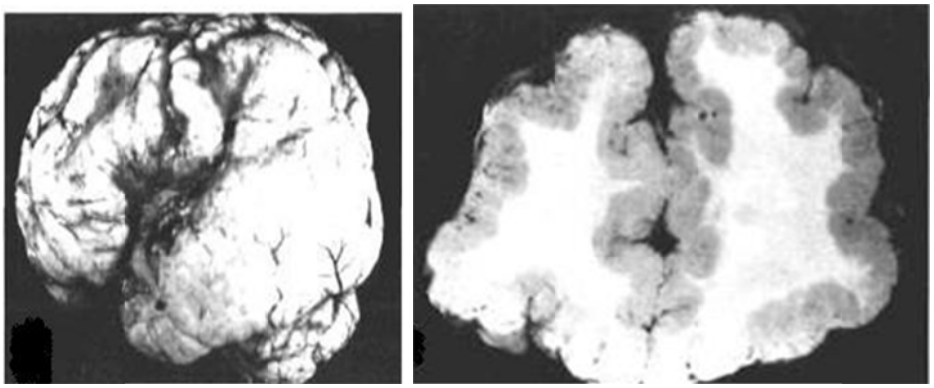


Figure 24. Gross appearance of the brain in a patient with FCMD.

Cloudy thickened leptomeniges over the frontal lobes are present. Coronal section of the brain in the same patient shows focal fusion of the medial surfaces of the hemispheres.

Adapted from Takada *et al.* 1984 (300) with permission.

Overlap of Dystroglycanopathy Phenotypes

Several papers reported the overlapping brain pathology seen in WWS, MEB and FCMD. Formal attempts to separate the conditions have suggested that WWS is the most severe, most often associated with large areas of complete lissencephaly and severe structural abnormalities such as encephalocele or fused cortex and profound abnormalities of the cerebellum. MEB does not have the severe structural abnormalities seen in WWS but often exhibits the remaining features, including cobblestone complex, ventricular dilatation, white matter changes, brainstem hypoplasia, cerebellar hypoplasia, dysplasia and cysts, but to a lesser degree of severity. FCMD is typically viewed as a milder version of MEB/WWS although reports of encephalocele and focal cortical fusion in FCMD exist and it is associated with breaches of the pial membrane found in cobblestone complex(250).

Cobblestone Complex like Variants

Autosomal Recessive Bilateral Frontoparietal Polymicrogyria (BFPP) is caused by mutations in *GPR56*, a G-protein coupled receptor (301). Patients with BFPP typically have mild to moderate mental retardation, motor developmental delay, seizures and non-progressive cerebellar signs. A neuromuscular phenotype has not been reported. MRI brain changes include BFPP, bilateral white matter change and hypoplasia of the cerebellum and pons (302). GPR56 has a role in organisation of pial membrane and regulation of anchorage of radial glial endfeet (303). The overmigration of neurones through the basement membrane in patients with GPR56 mutations results in a cobblestone cortex. Similarly in the cerebellum, granule cells show loss of adhesion to ECM molecules of the basement membrane (304). Both of these processes resemble that seen in the dystroglycanopathies (Figure 20).

Autosomal recessive Debré type cutis laxa (ARCL2) is a rare disorder characterised by developmental delay, facial dysmorphism and lax skin with myopia, strabismus and seizures reported in some individuals. In addition to the clinical similarities, MRI brain findings show some overlap with those seen in dystroglycanopathies, in particular cortical malformation involving the posterior frontal, perisylvian and parietal areas, partly resembling polymicrogyria. Some patients also have cerebellar hypoplasia. It is due to mutations in *ATP6V0A2*, a gene encoding the a2 subunit of the V-type H+ ATPase that has an important role in Golgi function. The mutations result in abnormal glycosylation of serum proteins (CDG-II) and result in defective Golgi trafficking in

fibroblasts from affected individuals. As highlighted previously (section 1.6.8) in reference to DPM3 these findings reiterate the similarity between congenital disorders of glycosylation and the dystroglycanopathies (305, 306).

Sprecher *et al.* described in 2005 a new condition in 7 individuals from 2 consanguineous Arab families, comprising cerebral dysgenesis, neuropathy, ichthyosis and palmoplantar keratoderma (CEDNIK syndrome). It was found to be a result of mutations in *SNAP29*, a gene that encodes a SNARE protein involved in vesicle trafficking and fusion. The cerebral abnormalities are similar to those seen in dystroglycanopathies and include cortical dysplasia with pachygyria and polymicrogyria and various degrees of corpus callosum abnormalities (307).

4.1.1.6 Genotype Phenotype Correlations

The MRI brain changes seen in patients with mutations in FKRP has previously been reported (258, 264). The spectrum is very wide ranging from normal cognitive development and normal brain MRI, as seen in the patients described when the first *FKRP* mutations were identified (128, 205) to severe structural brain abnormality in patients with associated learning difficulties. These include a hierarchical pattern of changes from isolated cerebellar cysts (211, 308) to more severe structural involvement affecting the brainstem and pons and also polymicrogyria and cobblestone lissencephalic changes indistinguishable from those found in MEB and WWS, affecting fronto-parietal regions more than the occipital and temporal regions(207, 258). Ventricular dilatation and white matter abnormalities were also variably present in these patients (258, 264, 308).

More recently, it has become obvious that mutations in the other genes encoding known or putative glycosyltransferases can also be associated with variable brain involvement. Mutations in *POMT1* and *POMT2,* originally associated with WWS phenotype (130, 132), were recently associated with milder brain involvement such as in patients with cerebellar hypoplasia (210, 251, 252, 309) or even associated with normal brain MRI in patients with microcephaly and mild mental retardation (310). Similarly, mutations in *FKTN,* originally identified in Japanese patients with Fukuyama CMD, typically associated with severe structural brain changes including polymicrogyria-pachygyria, occasional hemispheric fusion, cerebellar cystic lesions and transient dysmyelination (255), have been reported in a few patients with clinical and brain imaging features almost identical to those previously identified in WWS (208,

226). More recently we and others described several families with milder allelic mutations in *FKTN* associated with normal intelligence and brain MRI (32, 231). In contrast, mutations in *POMGNT1*, originally identified in Finnish and Turkish patients with MEB (129), have so far been described in patients with clinical and imaging features evocative of a MEB phenotype (254, 259). Mutations in *LARGE* were initially reported in a patient with fronto-parietal pachygyria and brainstem hypoplasia (131), and have more recently been associated with WWS-like features (231, 311).

The aim of this study is to report brain MRI findings in 27 patients with muscular dystrophy and mutations in *POMT1, POMT2 , POMGNT1, FKTN and LARGE* in order to establish the spectrum of brain involvement associated with each individual gene as well as any genotype-phenotype correlations. Scans of patients with mutations in FKRP were not included as the range of brain involvement in patients with mutations in this gene has previously been documented (258).

4.2 METHODS AND PATIENTS

Twenty-seven patients with mutations in *POMT1, POMT2, POMGNT1, FKTN* or *LARGE* were selected as they had MRI brain scans available for review. All but 6 of these patients are reported in Godfrey *et al.* (231) and as such were recruited on the basis of hypoglycosylation of alpha-dystroglycan (ADG) on muscle biopsy or clinical features highly evocative of α-dystroglycanopathy. Four of the remaining 6 patients (13, 14, 23 and 26) have reduction of ADG evident on skeletal muscle biopsy and the remaining 2 patients (18 and 20) have a phenotype suggestive of a dystroglycanopathy (Table 11). Details of genetic and pathological methods are as described in Godfrey *et al*.(231). Patients with *FKRP* mutations were excluded from this study as we have previously reported the spectrum of brain involvement, however their findings are summarised for completion in Table 13 (258).

All patients had undergone a brain MRI scan by the time the mutations were detected. Imaging studies were reviewed by at least two investigators who were blinded to clinical information and results of molecular genetic testing. The scans were reviewed using a proforma in which the following abnormalities were recorded: Infratentorial: cerebellar abnormalities (vermian and/or hemispheric involvement, including presence of cerebellar cysts or other signs of cerebellar dysplasia), shape and size of the brainstem and pons. Supratentorial: cortical malformation (severity and location), white

matter changes and ventriculomegaly. Any additional abnormalities were also recorded.

Patient	Gene	Mutations	CK	Phenotype	Microcephaly	Mental retardation	Eye involvement
1	*FKTN*	p.Arg307Gln p.Arg307Gln	3200	CMD	-	-	-
2	*FKTN*	p.Ala114Thr p.Thr287fs	9000	LGMD	-	-	-
3	*FKTN*	p.Arg307Gln p.Phe390fs	13000	LGMD	-	-	-
4	*FKTN*	p.Phe390fs p.Asp455fs	60000	LGMD	-	-	-
5	*POMT2*	p.Thr184Met p.Trp748Ser	1900	LGMD	-	+	-
6	*POMT1*	p.Phe173_Asn175delinsAsp p.Arg623Thr	8000	LGMD	+	+	-
7	*POMT1*	p.Tyr616del p.Gly65Arg	7800	CMD	N/A	+	-
8	*POMT1*	p.Glu143X p.Ala200Pro	18000	CMD	+	+	-
9	*POMT2*	p.Gly353Ser p.Gly726Glu	2000	CMD	+	+	Myopia

Patient	Gene	Mutations	CK	Phenotype	Microcephaly	Mental retardation	Eye involvement
10	*POMT2*	p.Phe117Ser p.Gly726Glu	5500	CMD	+	+	-
11	*POMGNT1*	c.652+1G>A p.Cys490Tyr	1000	CMD	-	+	Optic atrophy. Myopia
12	*POMT2*	p.Thr683Pro p.Ala351fs	3100	CMD	+	+	-
13	*POMT2*	p.Trp495Ser p.Trp748Arg	2900	CMD	+	+	-
14	*LARGE*	p.Glu509Lys p.667fs	4500	CMD	-	+	Nystagmus, abnormal ERG
15	*POMT1*	p.Ser727fsX p.Ser727fsX	3500	CMD	+	+	Congenital glaucoma
16	*POMT2*	p.Tyr666Cys p.Arg413Pro	N/A	CMD	N/A	N/A	Hypermetropia
17	*POMT2*	p.Ile198Asn p.Tyr666Cys	N/A	CMD	-	+	Myopia
18	*LARGE*	p.Gln87fs p.Ser331Phe	400	CMD	-	+	Myopia

Patient	Gene	Mutations	CK	Phenotype	Microcephaly	Mental retardation	Eye involvement
19	*POMT2*	p.Val373Phe p.Ile198Asn	6000	CMD	+	+	Congenital cataracts
20	*POMGNT1*	c.1539+1G>A p.Arg580X	740	CMD	-	+	Myopia, retinal dysplasia
21	*POMGNT1*	p.Arg367His c.1539+1G>A	2800	CMD	N/A	+	Ptosis, retinal atrophy
22	*POMGNT1*	p.Thr176Pro p.Thr176Pro	780	CMD	N/A	+	Congenital glaucoma
23	*POMGNT1*	p.Asp427His p.Asp150fsX	1500	CMD	+	+	Myopia
24	*POMGNT1*	c.1539+1G>A c.1539+1G>A	1200	CMD	+	+	Retinal detachment
25	*POMGNT1*	p.Trp475X p.Trp475X	1300	CMD	N/A	+	-
26	*POMT2*	p.Thr433X p.Thr433X	3200	CMD	-	+	Cataracts, bupthalmus
27	*LARGE*	p.Trp516X -	5700	CMD	N/A	+	Retinal dysplasia

Table 11. Gene mutations and Clinical Phenotype.

CMD; congenital muscular dystrophy, LGMD; limb girdle muscular dystrophy, ERG; electroretinogram.

4.3 RESULTS

Twenty-seven patients fulfilled the inclusion criteria; all but one had 2 allelic mutations in one of the genes studied. One patient had a single pathogenic *LARGE* mutation; she had typical WWS phenotype and died in the first few months of life (patient 27) (231) . Four patients had *FKTN* mutations, 4 patients had *POMT1* mutations, 9 patients had *POMT2* mutations, 7 patients had *POMGNT1* mutations and 3 patients had *LARGE* mutations. All patients had a CMD phenotype with the exception of 5 patients with an LGMD phenotype. The age at brain MRI scan ranged from 3 weeks to 16 years (mean age 4.04 years; 11 scans were of patients younger than 2 years of age). Only 4 patients were reported to have normal cognitive development but this was not always formally tested (Table11).

The MRI findings are summarised in Table12. In summary, 8 patients had normal brain scans (cases 1-3) or minimal changes (cases 4-8) such as periventricular white matter changes and/or mild ventricular dilatation. Two patients (cases 9,10) had evidence of cerebellar hypoplasia with no cortical abnormality. Two other patients (cases 11,12) had cerebellar cysts or hypoplasia and pontine abnormalities but no obvious cortical changes. Two patients (cases 13,14) had fronto-parietal-temporal polymicrogyria but no involvement of the cerebellum or brainstem (Figure 25). Three patients (cases 15-17) had cortical abnormalities with cerebellar dysplasia and posterior concavity abnormality of the brainstem (two out of three) but a normal appearance to the pons. Eight patients (cases 18-25) showed changes consistent with an MEB phenotype (Figure 26, 27 and 28). The remaining 2 patients (cases 26-27) had features consistent with a WWS phenotype.

Brainstem abnormalities were seen in a total of 14 patients. A thin brainstem with flattening of the pons was common, occurring in 13 of 27 scans including those without cortical involvement. In the more abnormal scans the brainstem had an unusual concavity to the posterior aspect best seen when viewed in sagittal sections (Figure 26). In 5 patients there was an abnormally thin brainstem with ventral and dorsal clefts of the pons (Figure 26 and 28). This was associated with an abnormal appearance to the corticospinal tracts with excessive areas of rounded short T1 and short T2 (Figure 28). Three of these patients had *POMGNT1* mutations (cases 20,21 and 25), 1 patient had a *POMT2* mutation (case 19) and 1 patient had a *LARGE* mutation (case 18).

Pt	Age at scan	Gene	VD	White matter changes	Cerebellar cysts	Other cerebellar abnormality	Brainstem abnormality	Pontine abnormality	Cortical abnormality
1	5Y	*FKTN*	-	-	-	-	-	-	-
2	10Y	*FKTN*	-	-	-	-	-	-	-
3	3Y	*FKTN*	-	-	-	-	-	-	-
4	8Y	*FKTN*	+	-	-	-	-	-	-
5	6Y	*POMT2*	±	-	-	-	-	-	-
6	3Y	*POMT1*	-	PV	-	-	-	-	-
7	2Y	*POMT1*	-	PV	-	-	-	-	-
8	7Y	*POMT1*	+	PV	-	-	-	-	-
9	3Y	*POMT2*	-	PV	-	Hypoplastic	-	-	-

Pt	Age at scan	Gene	VD	White matter changes	Cerebellar cysts	Other cerebellar abnormality	Brainstem abnormality	Pontine abnormality	Cortical abnormality
10	3Y	*POMT2*	+	PV	+	Hypoplastic	-	Hypoplasia	-
11	16Y	*POMGNT1*	+	Diffuse abnormality	+	-	-	Hypoplasia	-
12	9M	*POMT2*	-	PV and FP	-	Hypoplastic vermis	Posterior concavity	-	-
13	3M	*POMT2*	-	-	-	-	-	-	FPT PMG
14	14Y	*LARGE*	-	PV, T	-	-	Posterior concavity	Hypoplasia	FP pachygyria
15	3W	*POMT1*	++	TO>FP	+	Dysplastic vermis	Posterior concavity	-	TO PMG
16	1Y	*POMT2*	+	FP Reduced WM	+	Dysplastic vermis.	Posterior concavity	-	Focal PMG (A>P)
17	13Y	*POMT2*	-	PV	+	Dysplastic vermis	-	-	Posterior PMG
18	1Y	*LARGE*	+	Abnormal	+	Dysplastic vermis	Posterior concavity	Hypoplasia and cleft	FP PMG

Pt	Age at scan	Gene	VD	White matter changes	Cerebellar cysts	Other cerebellar abnormality	Brainstem abnormality	Pontine abnormality	Cortical abnormality
19	3Y	*POMT2*	++	Diffuse abnormality	+	Cerebellar cleft	Posterior concavity	Hypoplasia, cleft and abnormal corticospinal tract	FP PMG
20	5M	*POMGNT1*	++	Abnormal	++	Dysplastic vermis	Posterior concavity	Hypoplasia, cleft and abnormal corticospinal tract	Diffuse PMG, slight FP gradient
21	3Y	*POMGNT1*	++	Abnormal foci	+	Dysplastic	Posterior concavity	Hypoplasia, cleft and abnormal corticospinal tract	FP pachygyria,PMG T lobes, cobblestone cortex
22	5M	*POMGNT1*	++	Reduced WM	+	Hypoplastic vermis	Posterior concavity	Hypoplasia	Frontal pachygyria, cyst in T pole
23	8M	*POMGNT1*	++	Diffuse abnormality	++	Dysplastic, hypoplastic	Posterior concavity	Hypoplasia	FPT PMG
24	N/A	*POMGNT1*	++	Diffuse abnormality	+	Dysplastic	Hypoplasia	Hypoplasia	PMG (A>P) and pachygyria
25	18M	*POMGNT1*	+++	Diffuse abnormality	+	Dysplastic, hypoplastic	Anterior concavity	Hypoplasia, cleft and abnormal corticospinal tract	Diffuse PMG
26	5W	*POMT2*	+++			Hypoplastic	Hypoplasia	Hypoplasia	Thin lissencephalic cortical mantle

Pt	Age at scan	Gene	VD	White matter changes	Cerebellar cysts	Other cerebellar abnormality	Brainstem abnormality	Pontine abnormality	Cortical abnormality
27	4W	*LARGE*	++ +	Diffuse abnormality		Dyplastic, hypoplastic	Posterior concavity	Hypoplasia	Posterior lissencephaly, cobblestone variant

Table 12. Brain MRI findings.

Pt; patient, VD; ventricular dilatation, + indicates presence, - indicates absence of abnormality, F; frontal, T; temporal, O; occipital, P; parietal, A>P; anterior> posterior, PV; periventricular, WM; white matter, PMG; polymicrogyria.

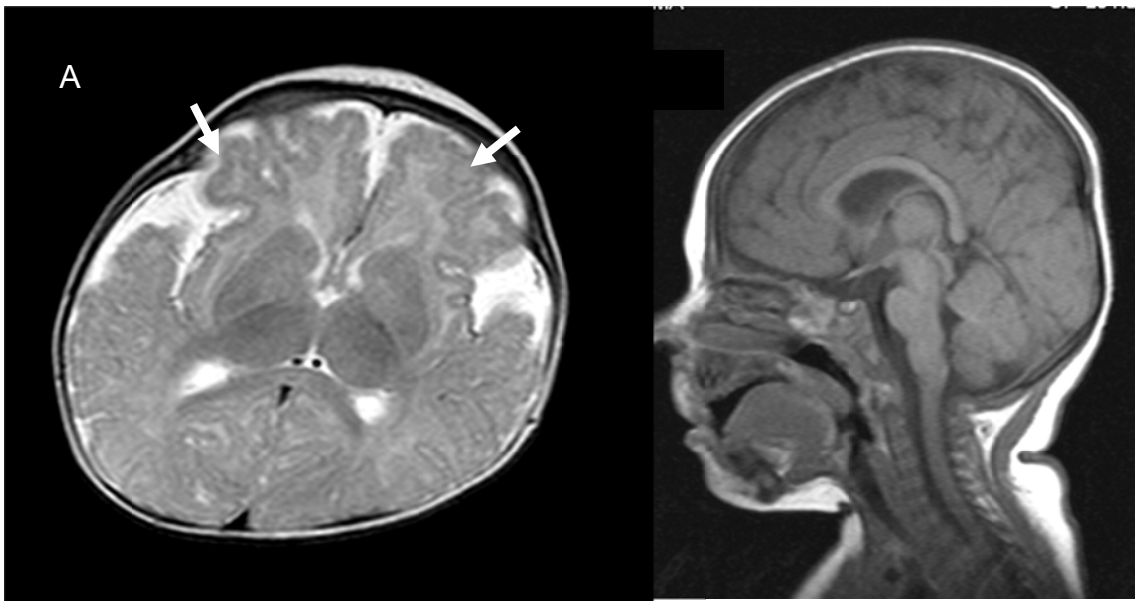


Figure 25. MRI brain scan of 3 month old with POMT2 mutations (case 13).

(A) T2- weighted image in the transverse plane. Note the predominant frontal cortical polymicrogyria (arrows).
(B) T1- weighted image in the sagittal plane showing the normal appearance of pons, brainstem and cerebellar vermis.

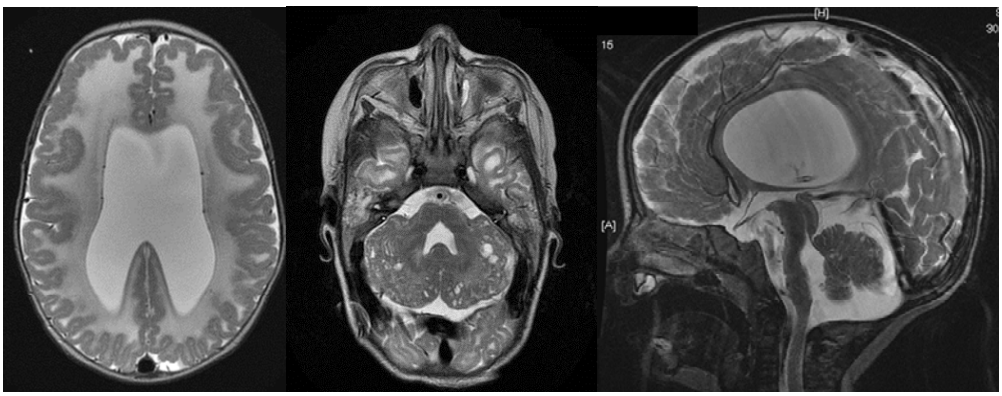


Figure 26. T2-weighted images in transverse and sagittal planes of a 5 month old (case 19) with POMGNT1 mutations.

There is widespread polymicrogyria in frontal and parietal lobes and abnormal high signal intensity throughout the white matter. There are multiple cysts in the hypoplastic cerebellar hemispheres in the transverse view. There are clefts in the dorsal and ventral pons. In the sagittal plane the pons is thinned with a concave posterior border.

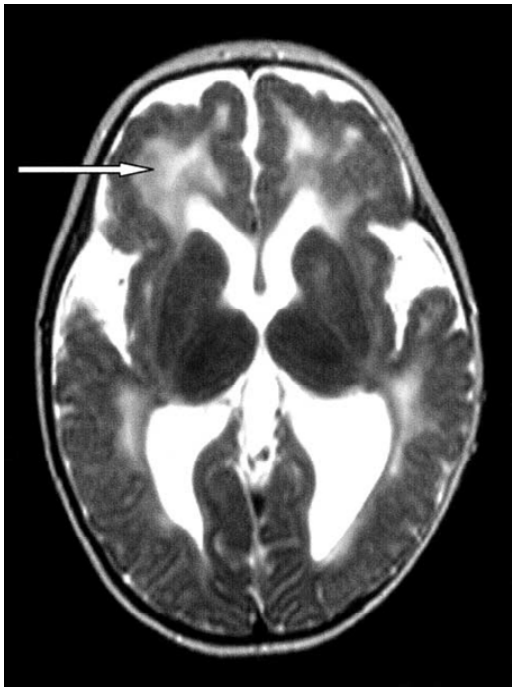


Figure 27. T2-weighted image in the transverse plane of an 8 month old (case 22) with heterozygous POMGNT1 mutations.

There is widespread bilateral polymicrogyria affecting predominantly the frontal and parietal lobes. The white matter is reduced in volume with abnormally high signal intensity. Areas of lower signal intensity, mainly within the anterior white matter (arrow), may represent heterotopic cells that have failed to migrate to the correct location.

Figure 28. T1 weighted image in the transverse plane of a 3 year old (case 20) with heterozygous POMGNT1 mutations.

There is a small ventral cleft in the pons. There is an exaggerated rounded appearance to the high signal myelinated corticospinal tracts (thin black arrows) There are several cerebellar cysts (thick black arrow).

4.3.1 Genotype Phenotype Correlation.

The 4 patients with *FKTN* mutations all had a normal MRI or minimal ventricular dilatation. Six of 7 patients with *POMGNT1* mutations had changes consistent with MEB and the remaining patient had cerebellar cysts and a flat pons but no obvious signs of cortical dysplasia on a scan performed at 9 months.

Three of the 4 patients with *POMT1* mutations had minimal periventricular white matter changes and one had cerebellar cysts and cortical changes but a normal pons.

The 9 patients with *POMT2* mutations had abnormalities which ranged from mild ventricular dilatation to isolated cerebellar hypoplasia to generalised cobblestone lissencephaly, resembling WWS.

The 3 patients with *LARGE* mutations had both diffuse cortical and white matter changes; one had cerebellar cysts and features entirely consistent with a MEB-like disorder; in one the severity of the lissencephaly was consistent with a WWS diagnosis. These results are summarised in Table13.

	Normal MRI (n=3)		**Normal cortex and cerebellum. Minimal VD+/-PV changes (n=5)**		**Cerebellar hypoplasia no cortical abnormalities (n=2)**		**Cerebellar cysts or hypoplasia, no cortical changes (n=2)**		**WM cortical changes. Cerebellum normal (n=2)**		**Cortex and cerebellar abnormality, normal pons (n=3)**		**MEB-like changes (n=8)**		**WWS-like changes (n=2)**	
	Our cohort	Other studies	Our cohort	Other studies	Our cohort	Other studies	Our cohort	Other studies	Our cohort	Other studies	Our cohort	Other studies	Our cohort	Other studies	Our cohort	Other studies
***FKTN* (n=4)**	●●●	**(126)**	●	**(126)**										**(300)**		**(208)**
***POMT1* (n=4)**		**(125, 251, 252, 312)**	●●●			**(251, 312)**					●			**(251)**		**(130, 251)**
***POMT2* (n=9)**			●		●●	**(210, 309)**	●		●		●●		●		●	**(132)**
***POMGNT1* (n=7)**							●						●●●●● ●	**(129, 254, 259)**		
***LARGE* (n=3)**									●	**(131)**			●		●	**(311)**
FKRP*		**(258)**		**(258)**				**(258)**		**(258)**				**(258)**		

Table 13. Distribution of MRI brain changes according to gene mutation.

Patients described in the present study are indicated as ●. The shaded areas indicate previously reported phenotypes, with the main references shown.

VD; ventricular dilatation, PV; periventricular, WM; white matter, WWS; Walker Warburg syndrome,

* *FKRP* not part of this study, results previously published

4.4 DISCUSSION

The findings reported in this study expand the spectrum of structural brain involvement associated with mutations in several of the genes involved in dystroglycanopathies and show that the range of central nervous system involvement due to individual gene defects is much wider than originally described.

The cortical abnormalities of patients with dystroglycanopathies has been described in multiple pathology reports and chapters. Three types of gross pathology have been described in the cerebral cortex of affected brains(297, 300, 313): (1) verrucose dysplasia in which nodules of cellular cortical tissue protrude through the pial basement membrane superficial to normally laminated cortex (chiefly in temporal lobes); (2) unlayered polymicrogyria with haphazardly oriented cortical neurons forming irregular clusters, separated by gliovascular strands extending from the pia (chiefly in frontal and parietal lobes) and (3) agyric regions with four distinctive layers (a-superficial layer containing myelinated fibers obscuring the molecular layer, b-thick cellular layer with disorganized and occasionally aggregated neurons, c-cell sparse layer of white matter, and d-heterotopic nodules of neurons). The agyric regions are located chiefly in the occipital lobes. Takada *et al*. noted that small superficial nodules are seen on the surface in all regions and suggested that the appearance seen on the surface of what are now called dystroglycanopathies is best called “cobblestone” cortex(300). It should be noted that both polymicrogyria and agyria are heterogeneous disorders. At least four different histological types of agyria have been described by Forman *et al*. (314, 315). Of note, these authors do not consider the agyric regions of dystroglycanopathies in their classification, instead classifying them separately as “cobblestone lissencephaly”. Here we have followed the nomenclature of Forman *et al*. Regarding polymicrogyria, while it is possible that many different histological subtypes of polymicrogyria can be identified, and some of these subtypes may also be separable by their imaging characteristics, such separation was not attempted in this study. We separated the cortical abnormalities identified in our patients into two broad categories, based upon the MRI appearance: polymicrogyria and cobblestone lissencephaly. Of note verrucose dysplasia cannot be detected by MRI. Polymicrogyria was diagnosed when either multiple microgyri were identified in the cerebral cortex on individual images or a slightly thickened (4-7 mm) cortex was seen, with irregularity of the cortical-white matter junction (316). Polymicrogyria has different appearances on MRI depending upon the state of myelination at the time of the scan(285). The individual microgyri are easily seen in the unmyelinated brain but, as myelination ensues, the

cortex begins to appear thick and can superficially resemble pachygyria. The two entities can be differentiated, however, by the lesser thickness (4-7 mm) and irregularity of the junction between the cortex and white matter in polymicrogyria (the cortex-white matter junction is smooth in pachygyria). Cobblestone lissencephaly was diagnosed when the cortex was thick (> 7 mm), the outer surface of the cortex was smooth, the inner surface of the cortex was irregular, and there was a layer of irregularly-shaped nodules of grey matter approximately 2 mm deep to the cortex. In many cases, these nodules appeared to be radially oriented immediately below a bundle of spindle-shaped grey matter in the cortex.

Three of the 27 patients studied had normal scans and another 5 had only mild changes such as minimal ventricular dilatation with periventricular white matter changes of long T2. These changes are non-specific and can be observed in low-risk full-term infants with normal developmental outcomes (317). The remaining 18 patients all had structural brain changes that almost invariably affected infratentorial structures, with the exception of one patient in whom only the fronto-parietal-temporal regions were polymicrogyric. In 4 of these 18 patients the infratentorial involvement was found in isolation, while in the remaining 14 patients it was associated with supratentorial cortical and white matter involvement. In this latter group of patients the supratentorial involvement ranged from diffuse periventricular white matter changes with focal areas of polymicrogyria to diffuse cortical structural abnormalities including cobblestone lissencephaly and an almost absent cortical mantle. In 2 cases, severe supratentorial findings were combined with abnormalities of the posterior fossa, consistent with the diagnosis of WWS (241). Infratentorial involvement included hypoplastic or dysplastic vermis, cerebellar cysts, a concavity in the brainstem at the floor of the fourth ventricle and pontine hypoplasia.

We correlated the results of the scans with the genotype of the patients, and compared them with the previously documented changes in *FKRP*-related dystrophies (207, 211, 258). Although some of the lesion patterns were more frequently associated with individual gene mutations, there was no unique defect which allowed unequivocal prediction of the primary gene defect. Florid cerebellar cysts were seen in all patients with *POMGNT1* mutations, but cysts were also found in 4 of 9 patients with *POMT2* mutations, in 1 of 4 patients with *POMT1* mutations and in 1 of 3 with *LARGE* mutations. To the best of our knowledge, cerebellar cysts have not been previously associated with any of these genotypes. Interestingly, cerebellar cysts in patients with *POMGNT1* mutations were also found in the absence of supratentorial white matter or cortical involvement, as we have previously reported for patients with *FKRP* mutations

(258), suggesting that the cerebellum is particularly vulnerable to the underlying disease process in patients with mutations in these two genes. Targeted screening of *POMGNT1* and *FKRP* should therefore be considered where cerebellar cysts are the predominant abnormality. In contrast, none of our patients with *FKTN* mutations had cerebellar cysts. All our patients with *FKTN* mutations were cognitively normal, in contrast to reports of individuals with FCMD where cerebellar polymicrogyria +/- cysts were found in 90% of patients (318). Increasingly it is possible to recognise a subgroup of *FKTN* associated phenotypes that are distinct from the classic FCMD reported in Japan, where learning difficulties and structural brain involvement are invariable. This is not surprising considering that virtually all Japanese patients carry the same founder mutation (127) a relatively severe mutation which has not been reported in non-Japanese patients. In patients with *FKTN* mutations, the spectrum of brain MRI findings, ranging from normal MRI (32) to the previously reported WWS-like changes(208, 226) resemble the spectrum we previously documented in patients with *FKRP* mutations (258).

Mutations in *POMT1, POMT2, POMGNT1* and *LARGE* were not associated with the same range of brain MRI findings reported for *FKRP*, and *FKTN.* Indeed, with the partial exception of *POMT2*, the other genes showed a relatively narrow range of findings; it is, however, of note that the patterns of lesions observed for an individual gene defect in this study are often different from those previously reported for the same gene. This was particularly evident for patients with *POMT2* mutations, originally associated with either a WWS or a cerebellar hypoplasia phenotype (132, 210); only 2 of the 9 cases we studied had these previously reported changes. In the other 7 cases the brain changes ranged from isolated mild ventricular dilatation or cerebellar dysplasia to various degree of cortical involvement, including isolated fronto-parieto-temporal polymicrogyria, which has not previously been reported. We also identified some patients with *POMGNT1,* and *POMT1* mutations with previously unreported findings such as isolated cerebellar cysts in *POMGNT1* and relatively mild cortical involvement without pons and brainstem abnormalities in *POMT1.* We also identified a patient with novel mutations in the *LARGE* gene, leading to a MEB-like picture with associated cerebellar cysts. It may well be that as further work and patients with mutations in these genes are identified, the spectrum of changes found in these three genes will also widen.

Other findings of this study were also of interest: in two patients the cortical changes affected predominantly the temporal and occipital regions. In one (patient 17, POMT2 mutation) the posterior cortical changes were associated with normal appearances to

the pons and brainstem, whilst in the other (patient 15, POMT1 mutation) the scan was performed at 3 weeks at an age when the extent and severity of cortical involvement cannot always be fully appreciated. The predominant posterior cortical involvement is at variance with the anterior-posterior gradient described in *FKRP* mutations (258) and *POMGNT1* (259, 319). Predominantly posterior cortical malformation is a feature of merosin negative CMD (320), while the co-existence of fronto-parietal polymicrogyria and occipital agyria, has been reported in FCMD patients (318).

We documented a ventral pontine cleft in 5 cases, 4 with a MEB-like condition (secondary to *POMGNT1* mutations in three and *LARGE* in another) and 1 with a more severe phenotype falling between the disease spectrum of MEB and WWS, carrying *POMT2* mutations. Such an abnormal appearance of the pons was reported at a similar time by Barkovich *et al.* in a number of patients with WWS and MEB(295). It is most likely caused by the absence of the ventral transverse pontine fibres, at the decussation of the middle cerebellar peduncles (295). Indeed these fibres migrate tangentially and their migration is impaired in the *myd*^{LARGE} mouse (288). MR diffusion tensor imaging studies may elucidate the exact position of pontine tracts and the structural basis for these abnormal imaging appearances (295).

The MRI changes reported here are highly evocative of a “dystroglycanopathy”. There is, however, an overlap with other conditions as mentioned previously. Patients with *GPR56* mutations have bilateral frontoparietal polymicrogyria seen in an anteroposterior distribution, bilateral patchy white matter changes and brainstem and cerebellar hypoplasia (282). Whilst the MRI findings are clearly similar to those seen in the midgroup of patients reported here (12-23), the polymicrogyria seen in patients with *GPR56* mutations affects predominantly the fronto-parietal regions and not the temporal and occipital areas also seen in patient with a dystroglycanopathy (302). In addition cerebellar cysts or scalloped appearance to the back of the brainstem has not been reported in patients with *GPR56* mutations.

A question that remains to be answered relates to whether or not patients exist with brain changes within the spectrum described with mutations in one of the dystroglycanopathy genes but with minimal /absent muscle involvement. We are aware of one patient with *POMGNT1* mutations in whom serum CK was normal, but ADG expression reduced on muscle biopsy(321). There are no systematic studies where the analysis of the putative or demonstrated glycosyltransferases mutated in dystroglycanopathies has been performed in patients without a skeletal muscle. phenotype.

In addition to looking for a correlation between different genes and phenotypes we also correlated the type of mutations with the brain MRI findings. Somewhat unsurprisingly the 3 scans with the most abnormal appearance (25,26,27) resulted from nonsense mutations but in 3 different genes, *POMGNT1*, *POMT2* and *LARGE*. Interestingly however, 2 patients with apparently severe mutations had less brain involvement than the 3 above. Patient 15 has a homozygous truncating mutation in *POMT1*, whilst patient 4 with no structural brain lesions was homozygous for two frameshifting mutations in *FKTN*. These findings may be explained by the location of these mutations in the most 3' exon, meaning that the mutant products may not be subject to nonsense mediated RNA decay(126).

A limitation of this study relates to the fact that the scans had been performed in various centres, at different ages of the patients, and not always optimised with thin (i.e. 1.5 mm) slices to detect subtle dysplastic changes, as most scans were 5 mm thick. Nevertheless these are the diagnostic scans routinely available in clinical settings; hence our findings are of relevance for this group of patients.

The reason for the varied abnormalities of brain involvement seen in patients carrying mutations in these different genes is not fully understood. All these gene products have so far only been involved in the glycosylation of ADG. The appropriate glycosylation of ADG plays a crucial role for its function as it regulates its binding to extracellular matrix proteins such as laminin, neurexin, perlecan and agrin (146). Although the role of ADG in brain development is not fully understood, it has been shown to be important for normal basement membrane formation and neuronal migration. As ADG is highly expressed in the pial membranes, it is likely that its abnormal glycosylation results in reduced integrity and the resulting breaches observed in these CMD variants (171), observations supported by the numerous studies performed in knockout mice. (291) (294) (146, 178, 291, 312).

In addition to the role of ADG in basement membrane stability, recent studies also suggest that it is involved in the process of migration of cerebellar neurons (290).

The difference observed in the pattern of brain involvement in patients carrying different gene mutations might suggest that some of these demonstrated or putative glycosyltransferases have targets other than ADG in brain, although this has never been demonstrated; another possibility is that it might reflect differences in the pattern of expression of individual glycosyltransferases in different brain regions. Less likely, it

may reflect a mutation specific effect on the ability of these glycosyltransferases to differentially bind with interacting proteins. Further work on the relevant animal models will help to clarify these issues.

CHAPTER 5. CONCLUDING DISCUSSION

5.1 THE BROADENING SPECTRUM OF CMD

The term Congenital Muscular Dystrophy encompasses a complex and heterogeneous group of disorders. They are broadly defined by an early clinical presentation with hypotonia, weakness and developmental delay with dystrophic appearance on muscle biopsy. Many have additional clinical features including contractures, brain and eye abnormalities, skin defects and raised CK. Although this definition is a good starting point, it is clear that many patients with CMD do not adhere to these criteria. Patients with genetically proven CMD may show no dystrophic abnormality on muscle biopsy and others with mutations in causative CMD genes present later, with features more consistent with LGMD. In a number of CMDs CK is normal or only mildly elevated. This is indeed somewhat unsatisfactory but in the absence of comprehensive molecular and genetic definitions, it remains a useful clinical framework on which to build.

In 2006 when this project started, the molecular and genetic basis for many forms of CMD was emerging. This allowed a rudimentary division of CMDs according to the cellular location of proteins involved. What is clear is that the majority of CMDs involve proteins localised to or interacting with the skeletal muscle ECM, reflecting the importance of this structure in prenatal muscle formation and postnatal maintenance of muscle integrity. The array of extra skeletal muscle manifestations seen in a number of conditions however are a testament to the expression of various CMD proteins in other organs and the result of diverse functions of the ECM in these.

The work presented in Chapter 2 reinforces the prevalence of ECM protein abnormalities in the UK population of CMD. Among the patients I reviewed fulfilling the diagnostic criteria for CMD, 46% had a confirmed molecular defect in a protein of the ECM (21% collagen VI related, 15% dystroglycanopathy, 10% MDC1A). Allowing for the fact that 50% of patients remained undiagnosed from a genetic perspective, this leaves a small proportion of patients with defects in genes of the nuclear envelope (EDMD2 3%) or endoplasmic reticulum (1%).

Whilst the ECM seems to be a prime location for proteins defective in CMDs, other groups of proteins are also known to be important. Lamin A/C until recently was the only nuclear envelope protein known to give rise to a CMD phenotype (although *emerin* mutations are known to give rise to X-linked EDMD). Work over the last few years has revealed that nesprin is also implicated in CMD phenotypes. CMD with adducted

thumbs(26) is due to mutations in *SYNE-1*, that encodes nesprin-1(25). Nesprin-1 mutations have also been associated with 2 other phenotypes; splice site mutations in *SYNE*-1 that result in loss of the KASH domain of nesprin-1 result in a form of autosomal recessive arthrogryposis with myopathic features and mutations in *SYNE-1* have also been found in ARCA1, a late onset autosomal recessive cerebellar ataxia(46, 322). Nesprin-1 has been shown to be involved in anchoring specialized myonuclei underneath neuromuscular junctions (323). It also binds Lamin A and emerin *in vitro* and *in vivo*. By binding to lamin and emerin, nesprins link the nucleoskeleton and inner nuclear membrane to the outer nuclear membrane and outer cytoskeleton (324). Nesprin-1 also belongs to the LINC complex, linking the nuclear lamina to the actin cytoskeleton in the cytoplasm, a role that conceivably may be important in the pathogenesis of CMD phenotypes(19). Interestingly, given nesprin-1s interaction with lamin A, the variable phenotype produced by mutations in the *SYNE-1* is reminiscent, although not as extreme, as the picture seen with LMNA mutations(22, 30, 115, 118).

This work in Chapter 2 also supports the notion that some subtypes of CMD are rare. We observed no cases of integrin α7 deficiency and there remains only 3 cases of this reported worldwide. Interestingly, CMD with hyperlaxity (CMDH) reported in the French Canadian population is now known to result from mutations in integrin α9 (ITGA9), confirming this as the second integrin (after integrin α7) to be implicated in the pathogenesis of CMD (26, 29). It remains to be seen whether other integrins may also be implicated in CMD.

We found no further cases of MDC1B in out cohort and the total number of reported cases of this stands at 6(28). Among the dystroglycanopathies, mutations in *LARGE* remain infrequent.

The work on the frequency of CMD variants also highlights the large number of disorders presenting clinically in a similar manner to CMD. Congenital myasthenia and CMD overlap in their clinical presentation, highlighted by the number of congenital myasthenia patients seen in our CMD referral population (222). Furthermore, it has come to light over that last couple of years that the CMD phenotype reported to be associated with 4p16.3 (Table 2) is in fact a congenital myasthenia with mutation in *DOK7* (F Muntoni, personal communication), highlighting just how similar these conditions may be (27). The complex interactions of the CMD proteins may go some way to explaining the phenotypic overlap with other conditions. DGC ligands in skeletal muscle include agrin (mutated in a form of limb girdle myasthenia) and BDG interacts

with rapsyn (mutated in a form of congenital myasthenia). Dystroglycan also has a role in stabilising acetylcholine receptors at the neuromuscular junction(141, 154, 158, 167).

The recent revelation that mutations in *DPM3* cause a phenotype with features consistent with both a dystroglycanopathy and congenital disorder of glycosylation is particularly interesting and has broadened the investigative approach when assessing patients with suspected CMD to include studying serum transferrin isoforms (44). Interestingly there has been speculation that one of the steps in the mannosylation of ADG is catalysed by a β1,4-galactosyltransferase (B4GalT). Mutations in *B4GalT* have been implicated in congenital disorder of glycosylation type IId (CDG IId; MIM 607091) again suggesting that these groups of disorders are more closely linked that previously anticipated (325, 326).

The continued progress in understanding the basis of CMD has resulted both in the widening spectrum of phenotypes attributed to mutations in many causative genes and in some cases the emergence of distinct subclasses of disorder. In 2010 it remains true that delineating the underlying aetiology of congenital muscular dystrophy and characterising the individual CMD phenotypes is a complex task.

5.2 DYSTROGLYCANOPATHIES

5.2.1 Genotype Phenotype Correlation

Work performed on identifying genotype phenotype correlations in the dystroglycanopathies in this thesis (Chapter 3) identified mutations in 34% of the cohort studied. Several new phenotypes were also identified including patients with *FKTN* and *POMGNT1* mutations without any mental retardation. The paper published documenting this work was the first of its kind and confirmed the heterogeneity seen in these disorders. Since this work was published (231), 3 further substantial dystroglycanopathy cohorts have been reported. In 2009 Mercuri *et al*. reported a large Italian cohort of patients including those with *FKRP* mutations. Mutations were detected in 43/81 (53%) and results confirmed the wide phenotypic spectrum associated with the dystroglycanopathies. *POMT1*, *POMT2* and *FKRP* mutations were the most frequently seen dystroglycanopathies in their population and MEB the most prevalent phenotype (327). They also reported variations on the classic dystroglycanopathy brain phenotypes with patients with isolated ventricular dilatation with no other findings reported with mutations in *POMT1* and *FKRP* and also 2 patients with isolated Chiari malformation type 1 with mutations in *POMT1* and *POMT2*. A cohort published by

Bouchet *et al.* reported the genetic mutations associated with foetal type 2 lissencephaly in the French population. They found mutations in 21/41 (53%) families with *POMT1* mutations the most prevalent, followed by *POMGNT1* and then *POMT2*. They did not find any likely pathogenic *FKRP*, *FKTN* or *LARGE* mutations in their patient population. Interestingly they found that *POMT1* and *POMT2* mutations consistently correlated with supra and infratentorial brain involvement whereas the patients with *POMGNT1* mutations were often more mildly affected (328). Manzini *et al*. published a study of 43 patients of varied ethnicity and geographical origin with WWS and found mutations in 40%. *POMT1* mutations were commonly found in patients from the Middle East whilst *FKTN* was a common cause of WWS in the patients of European descent. No mutations were found in *LARGE* or *POMGNT1 (329)*. Other smaller studies and case reports have also been published supporting our findings of *FKTN* as a frequent cause of LGMD with no mental retardation (227, 330). Mutations in *POMT2* have been reported in patients with LGMD with no mental retardation for the first time (LGMD2N) and also in patients with CMD with mental retardation (327, 331, 332) . A *FKTN* founder mutation has also been identified in the Ashkenazi Jewish population that causes WWS. The consistent message throughout these studies has been, with the exception of a few founder mutations, genetic and phenotypic heterogeneity is extensive in this group of disorders. It is also apparent that at least 50% of patients with a dystroglycanopathy phenotype have no mutations in *POMT1, POMT2, POMGNT1, FKRP, FKTN or LARGE*. The recent identification of CDG1o as a new dystroglycanopathy phenotype means that *DPM3* has not been excluded in many patients but it is unlikely to be the only gene contributing to the undiagnosed cases. Indeed collaborative studies with the Dutch group which described this condition has not identified DPM3 mutations in 20 dystroglycanopathy patients from our centre (Francesco Muntoni personal communication). The genotype phenotype associations for the dystroglycanopathy genes known in 2010 is summarised in Table 14.

Gene	WWS	MEB	FCMD	CMD-CRB	CMD - MR	CMD – no MR	LGMD - MR	LGMD – no MR	CMD 1X	CDG 1o
POMT1	(130, 251, 253)	(333)		(327)	(130, 251, 253)	(327)	(125, 252)			
POMT2	(132)	(210)		(333)	(327, 331)		(333)	(334)		
POMGNT1	(254)	(129, 254)						(333)		
FKTN	(208, 226, 335)	(333)	(127, 255)			(333)		(126, 227, 330)	(32)	
FKRP	(207)	(207)		(211)	(211)	(128)		(192)		
LARGE	(311, 327, 333)				(131)					
DPM3										(44)

Table14. The main dystroglycanopathy genotype-phenotype associations in 2010.

Blue shading indicates known associations before our study commenced, red indicates phenotypes identified by our study and green indicates subsequently identified phenotypes. Key references are indicated.

WWS; Walker Warburg Syndrome, MEB; Muscle Eye Brain Disease, FCMD; Fukuyama CMD, CMD-CRB; CMD with cerebellar involvement, CMD - MR; CMD with mental retardation, CMD –no MR; CMD without mental retardation, LGMD - MR; LGMD with mental retardation, LGMD –no MR; LGMD without mental retardation, CMD1X; dilated cardiomyopathy 1X, CDG1o; congenital disorder of glycosylation type 1o.

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5.2.2 Classification of Dystroglycanopathies

'it is increasingly clear that historical, eponymous classifications may represent an unreliable guide to the complex overlap of phenotypes generated by locus and allele heterogeneity'

Reproduced with permission Cormand *et al*. 2001(206).

This quote by Cormand *et al.* remains true 9 years later. Whilst the advances in the understanding of the molecular basis of CMDs has helped in so far as separating the main classes of CMD according to the location of the proteins involved, intragroup nomenclature, particularly in the dystroglycanopathies is unsatisfactory. The large number of individual case reports of phenotypes ascribed by us and others to dystroglycanopathy genes has led to many different names for dystroglycanopathy phenotypes. At the last count the following phenotypes had been reported: WWS, MEB, FCMD, MDC1B, MDC1C, MDC1D, LGMD2I, LGMD2K, LGMD2M, LGMD2N (actually assigned initially to 2 different conditions), Italian MEB, CMD with cerebellar cysts, CMD with cataracts; CMD with adducted thumbs; CDM1X and CDG1o. This does not take into account the various terms initially used to describe WWS. The subtle difference between these conditions and the increasing genetic heterogeneity has resulted in confusion among the intermediate phenotypes. With the exception of a few founder mutations (*FKTN* retrotransposal insertion (FCMD), *FKRP* C286A (LGMD2I) and *POMT1* A200P (LGMD2K)) it is apparent that a distinctive dystroglycanopathy phenotype is rarely going to be exclusively caused by only one gene. Our comprehensive genotype phenotype study has reinforced the notion that similar phenotypes are caused by more than one gene; FCMD overlaps with MEB caused by *POMGNT1* and *POMT2*, LGMD phenotypes with no mental retardation can also be caused by mutations in *POMGNT1*, *POMT2* and *FKTN*. I found in trying to study this collective group of conditions that multiple names, far from being useful often caused confusion. Phenotypes described in the literature often needed to be simplified to the core essentials in order to be able to apply them to larger studies. I found a large benefit to simplifying the core phenotypes to WWS, MEB, FCMD (although in reality this was never needed as we had no Japanese patients in any of our studies) and then CMD and LGMD with and without mental retardation, a strategy that has continued in some of the subsequent departmental papers(38). Account also has to be made for the CDG overlap phenotypes and these for the time being should be considered separately. Keeping the eponymous names for the severe phenotypes is justified

because of the striking features observed in these conditions, making them readily discernable from the milder conditions. Simplifying the dystroglycanopathies to core phenotypes is a valid strategy in other circumstances. Certainly conveying the range of phenotypes to a mass audience less familiar with the conditions is helped enormously by adopting this simplified approach which is applicable in everyday clinical practice and sufficient for most purposes. The one area in which a stricter definition of phenotype is still very important however is for those discreet genetically distinct dystroglycanopathy subtypes that are relatively common where long term outcome data can be generated with some degree of reliability and the possibility of therapeutic intervention is significant. This applies particularly to LGMD2I and FCMD where the phenotypes are largely homogenous and common as a result of founder mutations. In terms of whether to 'lump or split' this group of conditions, whilst the molecular and genetic data are still incomplete I strongly favour lumping except in the specific circumstances described above.

Much work following directly from the dystroglycanopathy genotype phenotype study was performed by Dr Caroline Godfrey in our laboratory. A natural step on from this was to search for new dystroglycanopathy genes. Several good candidate genes have been screened for mutations in a cohort of 37 patients many of whom remained undiagnosed at the end of the initial study. Genes screened included *GYLTL1B, β3GNT1, WWP1, GYG1*, *GYG2*, *MGAT5B* and *DAG1* (C. Godfrey personal communication). No clearly pathogenic mutations were detected in any of the genes. Whilst this does not exclude their involvement in the pathogenesis of dystroglycanopathy, mutations in these genes are clearly not common causes of human dystroglycanopathy.

Another departmental study stemming from the genotype phenotype dystroglycanopathy paper was led by Dr Jimenez-Mallebrera. Muscle pathology findings in 24 patients with dystroglycanopathy were analysed, seeking to establish whether or not a correlation existed between ADG depletion on skeletal muscle biopsy and clinical severity (336). Such a correlation was observed in patients with mutations in *POMT1*, *POMT2* and *POMGNT1*. This was not however seen in patients with mutations in *FKTN* or *FKRP;* for example, some patients with mild LGMD phenotypes had a severe depletion of ADG epitope staining. This study concluded that ADG depletion could not reliably be used as an indicator of clinical severity.

The work described in chapter 3 also formed the basis for the subsequent study of MRI brain findings in patients with dystroglycanopathy. In a similar fashion to the work on genotype phenotype correlation, this work established the varied MRI appearance in patients with confirmed mutations although some consistent and potentially diagnostically useful patterns were observed; *POMGNT1* mutations were always seen in association with florid cerebellar cysts on brain MRI for example, a finding that can also be observed in *FKRP* mutations. Cerebellar cysts were also seen in some patients with *POMT1*, *POMT2* and *LARGE* mutations but these were typically not as extensive. Our results suggest that targeted screening of *POMGNT1* and *FKRP* should be considered where cerebellar cysts are the predominant abnormality. In contrast to the typical MRI brain changes seen in patients with FCMD, the four MRIs that we reviewed with *FKTN* mutations were either normal or had minimal changes. This finding reinforces the concept of a subgroup of *FKTN* related conditions that are distinct from classic FCMD seen in Japan, where almost invariably patients carry a mutation unique to that population. We additionally found some patients with isolated temporo occipital cortical changes, a distribution more commonly seen in MDC1A.The overlap in MRI appearance between patients with *GPR56* mutations and bilateral frontoparietal polymicrogyria and some of the dystroglycanopathy scans reviewed was also noted. This highlights the importance of considering dystroglycanopathy as a differential diagnosis in these conditions especially given the wide spectrum of presentation. It will be interesting to see whether patients with isolated brain changes and minimal / absent muscle involvement are reported in due course as the MRI brain phenotype becomes more universally recognisable as representative of a dystroglycanopathy.

5.3 FUTURE PERSPECTIVES

5.3.1 Mutation Detection

Although the sensitivity of genetic testing has steadily improved, there are clear limitations to the mutation detection methods used in our study. In particular, large heterozygous deletions or duplications are likely to be missed using present techniques. In some patients variants of unknown significance have been detected that cannot be proven to be pathogenic using current methods. In others, a diagnosis may eventually be made as a result of new technology, in particular the emergence of the NMD chip for block testing neuromuscular disorders in affected individuals. The DNC is currently involved in a large European consortium based project aimed at designing, producing and validating novel DNA-chips for diagnosing neuromuscular diseases. Arrays will be developed with oligonucleotide probes designed to all known neuromuscular disease genes as well as a large panel of novel genes including a number of the candidate genes screened by Caroline Godfrey as detailed above. The results of the first round of screening for the NMD chip are pending and the first few large rearrangements have been detected using this technique (http://www.nmd-chip.eu/about).

A further advance in mutation detection technology is the advent of next generation sequencing. The use of this technology to sequence the entire genome still remains prohibitively expensive in most cases yet the use of targeted sequence capture approaches followed by parallel DNA sequencing offers a cost effective alternative. Either specific genomic regions or the entire exome (constituting approximately 5% of the genome) can be captured on microarray and sequenced (337). DNA from a subset of undiagnosed CMD patients will be submitted for exome sequencing as part of the UK10K Project at the Wellcome Trust Sanger Institute, Cambridge, http://www.uk10k.org/. The UK10K project is expected to uncover many rare genetic variants that are important in human disease, CMD included, giving a much deeper picture of genetics that can be applied to other studies both in the UK and around the world.

Although the NMD chip and other next generation sequencing technologies are very exciting advances in the field of CMD testing, the quantity of data generated by such methods and in turn the accurate interpretation of results is likely to present its own set of challenges.

5.3.2 Treatment of CMD and Therapeutic Trials

Until very recently the basis of treatment in CMD was not aimed at a cure but directed at symptomatic relief. Despite this, many of the interventions introduced have significantly altered the quality of life and natural history of this group of disorders. The introduction of non-invasive ventilation has significantly improved life expectancy and maximising nutrition has also been important (108, 232, 338). Other therapies including physiotherapy and surgery for scoliosis have improved quality of life(339, 340). It has only been in recent years with the improved knowledge and understanding of the molecular and genetic basis for this group of condition that a shift in direction towards trying to treat the underlying biochemical defects rather than physical symptoms has been possible.

Dystroglycanopathies

One therapeutic strategy considered in the dystroglycanopathies has resulted from work by Barresi *et al*. on adenovirus-mediated forced overexpression of *LARGE* in *myd*^{LARGE} mice. Such overexpression was shown to result in synthesis of glycan-enriched ADG with an increased affinity for its extracellular ligands resulting in improved skeletal muscle pathology. Importantly, overexpression of *LARGE* could also overcome the hypoglycosylation of ADG resulting from mutations in genes other than *LARGE*. Adenovirus-mediated *LARGE* gene transfer in *FKTN* and *POMGNT1* and *POMT1* deficient patient cell lines resulted in improved glycosylation of ADG and enhanced binding of laminin(199, 341).

A separate strategy has considered the up regulation of *Galgt2* (cytotoxic T-cell GalNAc transferase). Transgenic overexpression of *Galgt2* in skeletal muscle increased glycosylation of ADG and leads to increased expression of ECM proteins including laminin α4 and laminin α5 (342, 343).

More generic approaches to gene therapy, trialled in other disorders may also have an application in the dystroglycanopathies. Compounds such as PTC124 (Ataluren), currently under assessment in Duchenne muscular dystrophy clinical trials, that allow read through of stop codons may be of some use in dystroglycanopathy patients who carry a nonsense mutation. Alternatively it may be possible to exploit gene delivery

systems using adeno-associated viral (AAV) vectors as demonstrated in dystrophin deficient models(344).

An important point to consider in these cases is what aspects of the disease are likely to be improved by any therapeutic strategy. Most of the work to date has concentrated on enhanced glycosylation of ADG, binding to ligands and improved skeletal muscle pathology as a positive outcome measures. What remains to be seen is whether it is realistic to expect an improvement in the extraskeletal manifestations of dystroglycanopathies. For this reason it may be that prenatal intervention needs to be considered as a long term goal.

Collagen VI Related Disorders

Much of the work looking at therapeutic targets in UCMD has concentrated on the finding of myofiber apoptosis as an endpoint in collagen VI dysfunction. This is thought to result from premature breakdown of the mitochondrial permeability transition pore (mPTP)- controlled potential (345, 346). Cyclosporin A (CsA) inhibits the mPTP and has been shown to rescue muscle ultrastructural defects and decrease the number of apoptotic nuclei. In a study of 5 patients with collagen VI myopathy, a trial of oral CsA largely normalised mitochondrial dysfunction apoptosis and increased muscle regeneration (347). An analogue of CsA, Debio 025, which selectively inhibits cyclophilin D (rather than calcineurin and cyclophilins as in the case of CsA) has also been tested in *col6a1*$^{-/-}$ mice with positive findings including normalisation of mitochondrial function in skeletal muscle fibres, significant reduction in ultrastructural damage and decreased incidence of apoptosis in diaphragm muscle fibres(348, 349). Testing of a further compound, Omigapil, that binds to GAPDH and acts as an inhibitor of apoptosis has been shown to work effectively in 2 animal models of CMD; *col6a1*$^{-/-}$ (col VI deficiency) but also the *dy*^{W}/*dy*^{W} mouse (MDC1A) (350) .

MDC1A

The pathological changes observed in MDC1A have provided researchers with several avenues to explore in terms of disease modification. Firstly, in common with UCMD, increased apoptosis is a finding in laminin α2 deficient mice and fish. This is thought to perhaps be a consequence of membrane instability and decoupling of the muscle from the ECM(351, 352). In addition muscle fibrosis is a prominent feature.

Strategies looking at overcoming the apoptosis include overexpression of BCL2 (an antiapoptotic protein), resulting from crossing laminin α2 deficient mice and transgenic mice that overexpress muscle specific BCL2 (353). This resulted in improved lifespan and increased growth rate in affected mice. Doxycyclin, another inhibitor of apoptosis, has also been shown to improve lifespan and muscle pathology in *lama2dy-W* mice (354). As described above, Omigapil has also shown to be effective in the MDC1A mouse model (350).

Other work had looked at ways to re-establish the connection between ADG and the basement membranes which is lost in MDC1A. A minigene derived from Agrin (mini-agrin), delivered via an AAV vector appears to be able to bridge the muscle to basement membrane by binding laminin-511. Trials in MDC1A mice reported an ameliorated phenotype with improved motility, lifespan and muscle pathology (355).
The extensive fibrosis in MDC1A is associated with increased expression of transforming growth factor (TGF)-β, the expression of which is promoted by Angiotensin II. Angiotensin II type 1 receptor antagonist losartan has recently been demonstrated to reduce fibrosis in laminin deficient mice models (356).

Finally, the more dramatic approach of bone marrow transplant has also been tried in MDC1A mouse models. This led to an improved life span, skeletal muscle pathology, grip strength and respiratory function compared with wild type mice, a finding not seen in *mdx* mice. The reasons for this are not entirely clear but it is thought that it might reflect the basal lamina disruption seen in *dy* mice skeletal muscle which might be rectified by infusion of bone marrow derived cells that restore the laminin – merosin networks(357).

SEPN1 Related Disorders

Recent work by Arbogast *et al*. demonstrates that selenoprotein N plays a key role in prevention of oxidative stress damage in cultured muscle cells from patients with *SEPN1* null mutations(105). Selenoprotein N is also associated with the ryanodine receptor type 1 (RYR1), a calcium release channel modulated by redox reactions (358). In cultured myoblasts null for *SEPN1*, both intracellular oxidant activity and cytosolic calcium were increased (105). The similarity between the clinical and pathological phenotypes of RYR1- related central core disease and patients with *SEPN1* mutations suggest that there may be common disease mechanisms at work (359).

The susceptibility of *SEPN1* null cultured myoblasts to oxidative stress related cell death can be ameliorated by treating the cells with N-acetylcysteine (NAC)(105). NAC can also reduce protein oxidation in these cells. NAC may potentially be of therapeutic use in patients with *SEPN1*-related myopathies and clinical trials are planned (360). Another potential therapy for *SEPN1* related disorders would be AAV virus directed gene therapy as *SEPN*-1 related myopathies result from loss of gene function and the gene of a size that would potentially make this feasible (360).

5.4 CONCLUDING COMMENTS

In order to assess the efficacy of any clinical trial or therapeutic intervention, solid natural history data is important and much of the clinical data gathered in the process of producing this thesis has contributed to the development of clinical databases which form the foundation for on-going clinical research. Alongside this I have developed disease specific proformas for use in clinics to enable relevant and accurate data to be captured to facilitate long term research.

This thesis provides an overview of the classification, clinical and genetic aspects of CMD in 2010, with particular emphasis on the expanding field of dystroglycanopathies. Whilst much has been learnt over the last decade, further work is needed to refine the pathogenic mechanisms underlying these disorders in order to assist in the ultimate goal of achieving successful therapeutic intervention or cures for these devastating conditions.

REFERENCES

1. Mostacciuolo ML, Miorin M, Martinello F, Angelini C, Perini P, Trevisan CP. Genetic epidemiology of congenital muscular dystrophy in a sample from north-east Italy. Hum Genet 1996;97:277-279.
2. Muntoni F, Voit T. The congenital muscular dystrophies in 2004: a century of exciting progress. Neuromuscul Disord 2004;14:635-649.
3. Batten F. Three cases of myopathy, infantile type. Brain 1903;26:147-148.
4. Dubowitz V, Sewry CA. Muscle Biopsy a Practical Approach, Third ed: Saunders, 2007.
5. Dubowitz V. 22nd ENMC sponsored workshop on congenital muscular dystrophy held in Baarn, The Netherlands, 14-16 May 1993. Neuromuscul Disord 1994;4:75-81.
6. Dubowitz V. 41st ENMC International Workshop on Congenital Muscular Dystrophy 8-10 March 1996, Naarden, The Netherlands. Neuromuscul Disord 1996;6:295-306.
7. Dubowitz V. 50th ENMC International Workshop: congenital muscular dystrophy. 28 February 1997 to 2 March 1997, Naarden, The Netherlands. Neuromuscul Disord 1997;7:539-547.
8. Dubowitz V. 68th ENMC international workshop (5th international workshop): On congenital muscular dystrophy, 9-11 April 1999, Naarden, The Netherlands. Neuromuscul Disord 1999;9:446-454.
9. Tome FM, Evangelista T, Leclerc A, et al. Congenital muscular dystrophy with merosin deficiency. C R Acad Sci III 1994;317:351-357.
10. Helbling-Leclerc A, Zhang X, Topaloglu H, et al. Mutations in the laminin alpha 2-chain gene (LAMA2) cause merosin-deficient congenital muscular dystrophy. Nat Genet 1995;11:216-218.
11. Davies KE, Nowak KJ. Molecular mechanisms of muscular dystrophies: old and new players. Nat Rev Mol Cell Biol 2006;7:762-773.
12. Jimenez-Mallebrera C, Brown SC, Sewry CA, Muntoni F. Congenital muscular dystrophy: molecular and cellular aspects. Cell Mol Life Sci 2005;2:809-823.
13. Sanes JR. The basement membrane/basal lamina of skeletal muscle. J Biol Chem 2003;278:12601-12604.
14. Michele DE, Campbell KP. Dystrophin-glycoprotein complex: post-translational processing and dystroglycan function. J Biol Chem 2003;278:15457-15460.
15. Gruenbaum Y, Margalit A, Goldman RD, Shumaker DK, Wilson KL. The nuclear lamina comes of age. Nat Rev Mol Cell Biol 2005;6:21-31.
16. Burke B, Stewart CL. Life at the edge: the nuclear envelope and human disease. Nat Rev Mol Cell Biol 2002;3:575-585.
17. Gerace L, Blum A, Blobel G. Immunocytochemical localization of the major polypeptides of the nuclear pore complex-lamina fraction. Interphase and mitotic distribution. J Cell Biol 1978;79:546-566.
18. Lin F, Worman HJ. Structural organization of the human gene encoding nuclear lamin A and nuclear lamin C. J Biol Chem 1993;268:16321-16326.
19. Crisp M, Liu Q, Roux K, et al. Coupling of the nucleus and cytoplasm: role of the LINC complex. J Cell Biol 2006;172:41-53.
20. Reed UC. Congenital muscular dystrophy. Part I: a review of phenotypical and diagnostic aspects. Arq Neuropsiquiatr 2009;67:144-168.
21. Lisi MT, Cohn RD. Congenital muscular dystrophies: new aspects of an expanding group of disorders. Biochim Biophys Acta 2007;1772:159-172.

22. Mercuri E, Poppe M, Quinlivan R, et al. Extreme variability of phenotype in patients with an identical missense mutation in the lamin A/C gene: from congenital onset with severe phenotype to milder classic Emery-Dreifuss variant. Arch Neurol 2004;61:690-694.
23. Mahjneh I, Bushby K, Anderson L, et al. Merosin-positive congenital muscular dystrophy: a large inbred family. Neuropediatrics 1999;30:22-28.
24. Muntoni F, Taylor J, Sewry CA, Naom I, Dubowitz V. An early onset muscular dystrophy with diaphragmatic involvement, early respiratory failure and secondary alpha2 laminin deficiency unlinked to the LAMA2 locus on 6q22. Eur J Paediatr Neurol 1998;2:19-26.
25. Voit T, Cirak S, Abraham S, et al. Congenital muscular dystrophy with adducted thumbs, mental retardation, cerbellar hypoplasia and cataracts is caused by mutation of Enaptin (Nesprin-1): The third nucelar envelopathy with muscular dystrophy. Neuromuscul Disord 2007;17:833-834.
26. Tetreault M, Duquette A, Thiffault I, et al. A new form of congenital muscular dystrophy with joint hyperlaxity maps to 3p23-21. Brain 2006;129:2077-2084.
27. Sellick GS, Longman C, Brockington M, et al. Localisation of merosin-positive congenital muscular dystrophy to chromosome 4p16.3. Hum Genet 2005;117:207-212.
28. Brockington M, Sewry CA, Herrmann R, et al. Assignment of a form of congenital muscular dystrophy with secondary merosin deficiency to chromosome 1q42. Am J Hum Genet 2000;66:428-435.
29. Tetreault M, Thiffault I, Loisel L, et al. Mutations in the Integrin responsible for a congenital muscular dystrophy with hyperlaxity and their impact on normal cellular adhesion. In: ASHG; 2009; Honolulu, Hawaii, 2009.
30. Quijano-Roy S, Mbieleu B, Bonnemann CG, et al. De novo LMNA mutations cause a new form of congenital muscular dystrophy. Ann Neurol 2008;64:177-186.
31. D'Amico A, Petrini S, Parisi F, et al. Heart transplantation in a child with LGMD2I presenting as isolated dilated cardiomyopathy. Neuromuscul Disord 2008;18:153-155.
32. Murakami T, Hayashi YK, Noguchi S, et al. Fukutin gene mutations cause dilated cardiomyopathy with minimal muscle weakness. Ann Neurol 2006;60:597-602.
33. Nakanishi T, Sakauchi M, Kaneda Y, et al. Cardiac involvement in Fukuyama-type congenital muscular dystrophy. Pediatrics 2006;117:e1187-1192.
34. Geranmayeh F, Clement E, Feng LH, et al. Genotype-phenotype correlation in a large population of muscular dystrophy patients with LAMA2 mutations. Neuromuscul Disord;20:241-250.
35. Jones KJ, Morgan G, Johnston H, et al. The expanding phenotype of laminin alpha2 chain (merosin) abnormalities: case series and review. J Med Genet 2001;38:649-657.
36. Mercuri E, Muntoni F, Berardinelli A, et al. Somatosensory and visual evoked potentials in congenital muscular dystrophy: correlation with MRI changes and muscle merosin status. Neuropediatrics 1995;26:3-7.
37. Dubowitz V, Sewry C. Muscle Biopsy: A practical approach, 3 ed: Saunders, 2006.
38. Jimenez-Mallebrera C, Maioli MA, Kim J, et al. A comparative analysis of collagen VI production in muscle, skin and fibroblasts from 14 Ullrich congenital muscular dystrophy patients with dominant and recessive COL6A mutations. Neuromuscul Disord 2006;16:571-582.
39. Mercuri E, Yuva Y, Brown SC, et al. Collagen VI involvement in Ullrich syndrome: a clinical, genetic, and immunohistochemical study. Neurology 2002;58:1354-1359.

40. Mercuri E, Clements E, Offiah A, et al. Muscle magnetic resonance imaging involvement in muscular dystrophies with rigidity of the spine. Ann Neurol;67:201-208.
41. Astrea G, Schessl J, Clement E, et al. Muscle MRI in FHL1-linked reducing body myopathy. Neuromuscul Disord 2009;19:689-691.
42. Longman C, Mercuri E, Cowan F, et al. Antenatal and postnatal brain magnetic resonance imaging in muscle-eye-brain disease. Arch Neurol 2004;61:1301-1306.
43. Shorer Z, Philpot J, Muntoni F, Sewry C, Dubowitz V. Demyelinating peripheral neuropathy in merosin-deficient congenital muscular dystrophy. J Child Neurol 1995;10:472-475.
44. Lefeber DJ, Schonberger J, Morava E, et al. Deficiency of Dol-P-Man synthase subunit DPM3 bridges the congenital disorders of glycosylation with the dystroglycanopathies. Am J Hum Genet 2009;85:76-86.
45. Voit T, Parano E, Straub V, et al. Congenital muscular dystrophy with adducted thumbs, ptosis, external ophthalmoplegia, mental retardation and cerebellar hypoplasia: a novel form of CMD. Neuromuscul Disord 2002;12:623-630.
46. Attali R, Warwar N, Israel A, et al. Mutation of SYNE-1, encoding an essential component of the nuclear lamina, is responsible for autosomal recessive arthrogryposis. Hum Mol Genet 2009;18:3462-3469.
47. Hillaire D, Leclerc A, Faure S, et al. Localization of merosin-negative congenital muscular dystrophy to chromosome 6q2 by homozygosity mapping. Hum Mol Genet 1994;3:1657-1661.
48. Hayashi YK, Tezak Z, Momoi T, et al. Massive muscle cell degeneration in the early stage of merosin-deficient congenital muscular dystrophy. Neuromuscul Disord 2001;11:350-359.
49. Miyagoe-Suzuki Y, Nakagawa M, Takeda S. Merosin and congenital muscular dystrophy. Microsc Res Tech 2000;48:181-191.
50. Colognato H, Yurchenco PD. Form and function: the laminin family of heterotrimers. Dev Dyn 2000;218:213-234.
51. Sewry CA, Uziyel Y, Torelli S, et al. Differential labelling of laminin alpha 2 in muscle and neural tissue of dy/dy mice: are there isoforms of the laminin alpha 2 chain? Neuropathol Appl Neurobiol 1998;24:66-72.
52. Philpot J, Sewry C, Pennock J, Dubowitz V. Clinical phenotype in congenital muscular dystrophy: correlation with expression of merosin in skeletal muscle. Neuromuscul Disord 1995;5:301-305.
53. Vainzof M, Marie SK, Reed UC, et al. Deficiency of merosin (laminin M or alpha 2) in congenital muscular dystrophy associated with cerebral white matter alterations. Neuropediatrics 1995;26:293-297.
54. Dubowitz DJ, Tyszka JM, Sewry CA, Moats RA, Scadeng M, Dubowitz V. High resolution magnetic resonance imaging of the brain in the dy/dy mouse with merosin-deficient congenital muscular dystrophy. Neuromuscul Disord 2000;10:292-298.
55. Matsumura K, Yamada H, Saito F, Sunada Y, Shimizu T. Peripheral nerve involvement in merosin-deficient congenital muscular dystrophy and dy mouse. Neuromuscul Disord 1997;7:7-12.
56. Talts JF, Timpl R. Mutation of a basic sequence in the laminin alpha2LG3 module leads to a lack of proteolytic processing and has different effects on beta1 integrin-mediated cell adhesion and alpha-dystroglycan binding. FEBS Lett 1999;458:319-323.
57. Nissinen M, Helbling-Leclerc A, Zhang X, et al. Substitution of a conserved cysteine-996 in a cysteine-rich motif of the laminin alpha2-chain in congenital muscular dystrophy with partial deficiency of the protein. Am J Hum Genet 1996;58:1177-1184.

58. Guicheney P, Vignier N, Zhang X, et al. PCR based mutation screening of the laminin alpha2 chain gene (LAMA2): application to prenatal diagnosis and search for founder effects in congenital muscular dystrophy. J Med Genet 1998;35:211-217.
59. Pegoraro E, Marks H, Garcia CA, et al. Laminin alpha2 muscular dystrophy: genotype/phenotype studies of 22 patients. Neurology 1998;51:101-110.
60. Sewry CA, Naom I, D'Alessandro M, et al. Variable clinical phenotype in merosin-deficient congenital muscular dystrophy associated with differential immunolabelling of two fragments of the laminin alpha 2 chain. Neuromuscul Disord 1997;7:169-175.
61. Muntoni F, Guicheney P. 85th ENMC International Workshop on Congenital Muscular Dystrophy. 6th International CMD Workshop. 1st Workshop of the Myo-Cluster Project 'GENRE'. 27-28th October 2000, Naarden, The Netherlands. Neuromuscul Disord 2002;12:69-78.
62. Naom I, D'Alessandro M, Sewry C, et al. The role of immunocytochemistry and linkage analysis in the prenatal diagnosis of merosin-deficient congenital muscular dystrophy. Hum Genet 1997;99:535-540.
63. Vainzof M, Richard P, Herrmann R, et al. Prenatal diagnosis in laminin alpha2 chain (merosin)-deficient congenital muscular dystrophy: a collective experience of five international centers. Neuromuscul Disord 2005;15:588-594.
64. Ullrich O. Kongenitale, atonisch-sklerotische Muskeldystrophie. Monatsschr Kinderheilkd 1930:502-510.
65. Camacho Vanegas O, Bertini E, Zhang RZ, et al. Ullrich scleroatonic muscular dystrophy is caused by recessive mutations in collagen type VI. Proc Natl Acad Sci U S A 2001;98:7516-7521.
66. Hessle H, Engvall E. Type VI collagen. Studies on its localization, structure, and biosynthetic form with monoclonal antibodies. J Biol Chem 1984;259:3955-3961.
67. von der Mark H, Aumailley M, Wick G, Fleischmajer R, Timpl R. Immunochemistry, genuine size and tissue localization of collagen VI. Eur J Biochem 1984;142:493-502.
68. Bruns RR. Beaded filaments and long-spacing fibrils: relation to type VI collagen. J Ultrastruct Res 1984;89:136-145.
69. Chu ML, Pan TC, Conway D, et al. The structure of type VI collagen. Ann N Y Acad Sci 1990;580:55-63.
70. Chu ML, Mann K, Deutzmann R, et al. Characterization of three constituent chains of collagen type VI by peptide sequences and cDNA clones. Eur J Biochem 1987;168:309-317.
71. Zhang RZ, Sabatelli P, Pan TC, et al. Effects on collagen VI mRNA stability and microfibrillar assembly of three COL6A2 mutations in two families with Ullrich congenital muscular dystrophy. J Biol Chem 2002;277:43557-43564.
72. Engvall E, Hessle H, Klier G. Molecular assembly, secretion, and matrix deposition of type VI collagen. J Cell Biol 1986;102:703-710.
73. Kuo HJ, Maslen CL, Keene DR, Glanville RW. Type VI collagen anchors endothelial basement membranes by interacting with type IV collagen. J Biol Chem 1997;272:26522-26529.
74. Bruns RR, Press W, Engvall E, Timpl R, Gross J. Type VI collagen in extracellular, 100-nm periodic filaments and fibrils: identification by immunoelectron microscopy. J Cell Biol 1986;103:393-404.
75. Keene DR, Engvall E, Glanville RW. Ultrastructure of type VI collagen in human skin and cartilage suggests an anchoring function for this filamentous network. J Cell Biol 1988;107:1995-2006.

76. Bidanset DJ, Guidry C, Rosenberg LC, Choi HU, Timpl R, Hook M. Binding of the proteoglycan decorin to collagen type VI. J Biol Chem 1992;267:5250-5256.
77. Bonaldo P, Russo V, Bucciotti F, Doliana R, Colombatti A. Structural and functional features of the alpha 3 chain indicate a bridging role for chicken collagen VI in connective tissues. Biochemistry 1990;29:1245-1254.
78. Tillet E, Wiedemann H, Golbik R, et al. Recombinant expression and structural and binding properties of alpha 1(VI) and alpha 2(VI) chains of human collagen type VI. Eur J Biochem 1994;221:177-185.
79. Wiberg C, Hedbom E, Khairullina A, et al. Biglycan and decorin bind close to the n-terminal region of the collagen VI triple helix. J Biol Chem 2001;276:18947-18952.
80. Howell SJ, Doane KJ. Type VI collagen increases cell survival and prevents anti-beta 1 integrin-mediated apoptosis. Exp Cell Res 1998;241:230-241.
81. Ruhl M, Johannsen M, Atkinson J, et al. Soluble collagen VI induces tyrosine phosphorylation of paxillin and focal adhesion kinase and activates the MAP kinase erk2 in fibroblasts. Exp Cell Res 1999;250:548-557.
82. Ruhl M, Sahin E, Johannsen M, et al. Soluble collagen VI drives serum-starved fibroblasts through S phase and prevents apoptosis via down-regulation of Bax. J Biol Chem 1999;274:34361-34368.
83. Voit T. Congenital muscular dystrophies: 1997 update. Brain Dev 1998;20:65-74.
84. Muntoni F, Bertini E, Bonnemann C, et al. 98th ENMC International Workshop on Congenital Muscular Dystrophy (CMD), 7th Workshop of the International Consortium on CMD, 2nd Workshop of the MYO CLUSTER project GENRE. 26-28th October, 2001, Naarden, The Netherlands. Neuromuscul Disord 2002;12:889-896.
85. Lampe AK, Bushby KM. Collagen VI related muscle disorders. J Med Genet 2005;42:673-685.
86. Mercuri E, Lampe A, Allsop J, et al. Muscle MRI in Ullrich congenital muscular dystrophy and Bethlem myopathy. Neuromuscul Disord 2005;15:303-310.
87. Pepe G, Bertini E, Bonaldo P, et al. Bethlem myopathy (BETHLEM) and Ullrich scleroatonic muscular dystrophy: 100th ENMC international workshop, 23-24 November 2001, Naarden, The Netherlands. Neuromuscul Disord 2002;12:984-993.
88. Haq RU, Speer MC, Chu ML, Tandan R. Respiratory muscle involvement in Bethlem myopathy. Neurology 1999;52:174-176.
89. Mercuri E, Cini C, Counsell S, et al. Muscle MRI findings in a three-generation family affected by Bethlem myopathy. Eur J Paediatr Neurol 2002;6:309-314.
90. Lampe AK, Dunn DM, von Niederhausern AC, et al. Automated genomic sequence analysis of the three collagen VI genes: applications to Ullrich congenital muscular dystrophy and Bethlem myopathy. J Med Genet 2005;42:108-120.
91. Lamande SR, Morgelin M, Selan C, Jobsis GJ, Baas F, Bateman JF. Kinked collagen VI tetramers and reduced microfibril formation as a result of Bethlem myopathy and introduced triple helical glycine mutations. J Biol Chem 2002;277:1949-1956.
92. Lamande SR, Shields KA, Kornberg AJ, Shield LK, Bateman JF. Bethlem myopathy and engineered collagen VI triple helical deletions prevent intracellular multimer assembly and protein secretion. J Biol Chem 1999;274:21817-21822.
93. Pepe G, Giusti B, Bertini E, et al. A heterozygous splice site mutation in COL6A1 leading to an in-frame deletion of the alpha1(VI) collagen chain in an italian family affected by bethlem myopathy. Biochem Biophys Res Commun 1999;258:802-807.

94. Pan TC, Zhang RZ, Sudano DG, Marie SK, Bonnemann CG, Chu ML. New molecular mechanism for Ullrich congenital muscular dystrophy: a heterozygous in-frame deletion in the COL6A1 gene causes a severe phenotype. Am J Hum Genet 2003;73:355-369.
95. Baker NL, Morgelin M, Peat R, et al. Dominant collagen VI mutations are a common cause of Ullrich congenital muscular dystrophy. Hum Mol Genet 2005;14:279-293.
96. Pepe G, de Visser M, Bertini E, et al. Bethlem myopathy (BETHLEM) 86th ENMC international workshop, 10-11 November 2000, Naarden, The Netherlands. Neuromuscul Disord 2002;12:296-305.
97. Higuchi I, Suehara M, Iwaki H, Nakagawa M, Arimura K, Osame M. Collagen VI deficiency in Ullrich's disease. Ann Neurol 2001;49:544.
98. Brockington M, Brown SC, Lampe A, et al. Prenatal diagnosis of Ullrich congenital muscular dystrophy using haplotype analysis and collagen VI immunocytochemistry. Prenat Diagn 2004;24:440-444.
99. Clarke NF, Kidson W, Quijano-Roy S, et al. SEPN1: associated with congenital fiber-type disproportion and insulin resistance. Ann Neurol 2006;59:546-552.
100. Ferreiro A, Ceuterick-de Groote C, Marks JJ, et al. Desmin-related myopathy with Mallory body-like inclusions is caused by mutations of the selenoprotein N gene. Ann Neurol 2004;55:676-686.
101. Ferreiro A, Quijano-Roy S, Pichereau C, et al. Mutations of the selenoprotein N gene, which is implicated in rigid spine muscular dystrophy, cause the classical phenotype of multiminicore disease: reassessing the nosology of early-onset myopathies. Am J Hum Genet 2002;71:739-749.
102. Moghadaszadeh B, Petit N, Jaillard C, et al. Mutations in SEPN1 cause congenital muscular dystrophy with spinal rigidity and restrictive respiratory syndrome. Nat Genet 2001;29:17-18.
103. Flanigan KM, Kerr L, Bromberg MB, et al. Congenital muscular dystrophy with rigid spine syndrome: a clinical, pathological, radiological, and genetic study. Ann Neurol 2000;47:152-161.
104. Petit N, Lescure A, Rederstorff M, et al. Selenoprotein N: an endoplasmic reticulum glycoprotein with an early developmental expression pattern. Hum Mol Genet 2003;12:1045-1053.
105. Arbogast S, Beuvin M, Fraysse B, Zhou H, Muntoni F, Ferreiro A. Oxidative stress in SEPN1-related myopathy: from pathophysiology to treatment. Ann Neurol 2009;65:677-686.
106. Dubowitz V. Rigid spine syndrome: a muscle syndrome in search of a name. Proc R Soc Med 1973;66:219-220.
107. D'Amico A, Haliloglu G, Richard P, et al. Two patients with 'Dropped head syndrome' due to mutations in LMNA or SEPN1 genes. Neuromuscul Disord 2005;15:521-524.
108. Schara U, Kress W, Bonnemann CG, et al. The phenotype and long-term follow-up in 11 patients with juvenile selenoprotein N1-related myopathy. Eur J Paediatr Neurol 2008;12:224-230.
109. Bonne G, Di Barletta MR, Varnous S, et al. Mutations in the gene encoding lamin A/C cause autosomal dominant Emery-Dreifuss muscular dystrophy. Nat Genet 1999;21:285-288.
110. Fisher DZ, Chaudhary N, Blobel G. cDNA sequencing of nuclear lamins A and C reveals primary and secondary structural homology to intermediate filament proteins. Proc Natl Acad Sci U S A 1986;83:6450-6454.

111. Dahl KN, Kahn SM, Wilson KL, Discher DE. The nuclear envelope lamina network has elasticity and a compressibility limit suggestive of a molecular shock absorber. J Cell Sci 2004;117:4779-4786.
112. Brown SC, Piercy RJ, Muntoni F, Sewry CA. Investigating the pathology of Emery-Dreifuss muscular dystrophy. Biochem Soc Trans 2008;36:1335-1338.
113. Bonne G, Mercuri E, Muchir A, et al. Clinical and molecular genetic spectrum of autosomal dominant Emery-Dreifuss muscular dystrophy due to mutations of the lamin A/C gene. Ann Neurol 2000;48:170-180.
114. Bonne G, Yaou RB, Beroud C, et al. 108th ENMC International Workshop, 3rd Workshop of the MYO-CLUSTER project: EUROMEN, 7th International Emery-Dreifuss Muscular Dystrophy (EDMD) Workshop, 13-15 September 2002, Naarden, The Netherlands. Neuromuscul Disord 2003;13:508-515.
115. Vytopil M, Benedetti S, Ricci E, et al. Mutation analysis of the lamin A/C gene (LMNA) among patients with different cardiomuscular phenotypes. J Med Genet 2003;40:e132.
116. Krimm I, Ostlund C, Gilquin B, et al. The Ig-like structure of the C-terminal domain of lamin A/C, mutated in muscular dystrophies, cardiomyopathy, and partial lipodystrophy. Structure 2002;10:811-823.
117. Benedetti S, Menditto I, Degano M, et al. Phenotypic clustering of lamin A/C mutations in neuromuscular patients. Neurology 2007;69:1285-1292.
118. Mercuri E, Brown SC, Nihoyannopoulos P, et al. Extreme variability of skeletal and cardiac muscle involvement in patients with mutations in exon 11 of the lamin A/C gene. Muscle Nerve 2005;31:602-609.
119. Fidzianska A, Toniolo D, Hausmanowa-Petrusewicz I. Ultrastructural abnormality of sarcolemmal nuclei in Emery-Dreifuss muscular dystrophy (EDMD). J Neurol Sci 1998;159:88-93.
120. Sewry CA, Brown SC, Mercuri E, et al. Skeletal muscle pathology in autosomal dominant Emery-Dreifuss muscular dystrophy with lamin A/C mutations. Neuropathol Appl Neurobiol 2001;27:281-290.
121. Fidzianska A, Hausmanowa-Petrusewicz I. Architectural abnormalities in muscle nuclei. Ultrastructural differences between X-linked and autosomal dominant forms of EDMD. J Neurol Sci 2003;210:47-51.
122. Hayashi YK, Chou FL, Engvall E, et al. Mutations in the integrin alpha7 gene cause congenital myopathy. Nat Genet 1998;19:94-97.
123. Vachon PH, Xu H, Liu L, et al. Integrins (alpha7beta1) in muscle function and survival. Disrupted expression in merosin-deficient congenital muscular dystrophy. J Clin Invest 1997;100:1870-1881.
124. Muntoni F, Brockington M, Torelli S, Brown SC. Defective glycosylation in congenital muscular dystrophies. Curr Opin Neurol 2004;17:205-209.
125. Balci B, Uyanik G, Dincer P, et al. An autosomal recessive limb girdle muscular dystrophy (LGMD2) with mild mental retardation is allelic to Walker-Warburg syndrome (WWS) caused by a mutation in the POMT1 gene. Neuromuscul Disord 2005;15:271-275.
126. Godfrey C, Escolar D, Brockington M, et al. Fukutin gene mutations in steroid-responsive limb girdle muscular dystrophy. Ann Neurol 2006.
127. Kobayashi K, Nakahori Y, Miyake M, et al. An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. Nature 1998;394:388-392.
128. Brockington M, Blake DJ, Prandini P, et al. Mutations in the fukutin-related protein gene (FKRP) cause a form of congenital muscular dystrophy with secondary laminin alpha2 deficiency and abnormal glycosylation of alpha-dystroglycan. Am J Hum Genet 2001;69:1198-1209.

129. Yoshida A, Kobayashi K, Manya H, et al. Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. Dev Cell 2001;1:717-724.
130. Beltran-Valero de Bernabe D, Currier S, Steinbrecher A, et al. Mutations in the O-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker-Warburg syndrome. Am J Hum Genet 2002;71:1033-1043.
131. Longman C, Brockington M, Torelli S, et al. Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of alpha-dystroglycan. Hum Mol Genet 2003;12:2853-2861.
132. van Reeuwijk J, Janssen M, van den Elzen C, et al. POMT2 mutations cause alpha-dystroglycan hypoglycosylation and Walker-Warburg syndrome. J Med Genet 2005;42:907-912.
133. Hoffman EP, Brown RH, Jr., Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 1987;51:919-928.
134. Roberds SL, Leturcq F, Allamand V, et al. Missense mutations in the adhalin gene linked to autosomal recessive muscular dystrophy. Cell 1994;78:625-633.
135. Lim LE, Duclos F, Broux O, et al. Beta-sarcoglycan: characterization and role in limb-girdle muscular dystrophy linked to 4q12. Nat Genet 1995;11:257-265.
136. Noguchi S, McNally EM, Ben Othmane K, et al. Mutations in the dystrophin-associated protein gamma-sarcoglycan in chromosome 13 muscular dystrophy. Science 1995;270:819-822.
137. Nigro V, de Sa Moreira E, Piluso G, et al. Autosomal recessive limb-girdle muscular dystrophy, LGMD2F, is caused by a mutation in the delta-sarcoglycan gene. Nat Genet 1996;14:195-198.
138. Ervasti JM, Campbell KP. Membrane organization of the dystrophin-glycoprotein complex. Cell 1991;66:1121-1131.
139. Yoshida M, Ozawa E. Glycoprotein complex anchoring dystrophin to sarcolemma. J Biochem 1990;108:748-752.
140. Petrof BJ, Shrager JB, Stedman HH, Kelly AM, Sweeney HL. Dystrophin protects the sarcolemma from stresses developed during muscle contraction. Proc Natl Acad Sci U S A 1993;90:3710-3714.
141. Ibraghimov-Beskrovnaya O, Ervasti JM, Leveille CJ, Slaughter CA, Sernett SW, Campbell KP. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature 1992;355:696-702.
142. Ibraghimov-Beskrovnaya O, Milatovich A, Ozcelik T, et al. Human dystroglycan: skeletal muscle cDNA, genomic structure, origin of tissue specific isoforms and chromosomal localization. Hum Mol Genet 1993;2:1651-1657.
143. Durbeej M, Campbell KP. Biochemical characterization of the epithelial dystroglycan complex. J Biol Chem 1999;274:26609-26616.
144. Langenbach KJ, Rando TA. Inhibition of dystroglycan binding to laminin disrupts the PI3K/AKT pathway and survival signaling in muscle cells. Muscle Nerve 2002;26:644-653.
145. Spence HJ, Dhillon AS, James M, Winder SJ. Dystroglycan, a scaffold for the ERK-MAP kinase cascade. EMBO Rep 2004;5:484-489.
146. Moore SA, Saito F, Chen J, et al. Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy. Nature 2002;418:422-425.
147. Saito F, Moore SA, Barresi R, et al. Unique role of dystroglycan in peripheral nerve myelination, nodal structure, and sodium channel stabilization. Neuron 2003;38:747-758.

148. Durbeej M, Ekblom P. Dystroglycan and laminins: glycoconjugates involved in branching epithelial morphogenesis. Exp Lung Res 1997;23:109-118.
149. Jacobson C, Montanaro F, Lindenbaum M, Carbonetto S, Ferns M. alpha-Dystroglycan functions in acetylcholine receptor aggregation but is not a coreceptor for agrin-MuSK signaling. J Neurosci 1998;18:6340-6348.
150. Montanaro F, Gee SH, Jacobson C, Lindenbaum MH, Froehner SC, Carbonetto S. Laminin and alpha-dystroglycan mediate acetylcholine receptor aggregation via a MuSK-independent pathway. J Neurosci 1998;18:1250-1260.
151. Matsumura K, Chiba A, Yamada H, et al. A role of dystroglycan in schwannoma cell adhesion to laminin. J Biol Chem 1997;272:13904-13910.
152. Jung D, Yang B, Meyer J, Chamberlain JS, Campbell KP. Identification and characterization of the dystrophin anchoring site on beta-dystroglycan. J Biol Chem 1995;270:27305-27310.
153. Chung W, Campanelli JT. WW and EF hand domains of dystrophin-family proteins mediate dystroglycan binding. Mol Cell Biol Res Commun 1999;2:162-171.
154. Henry MD, Campbell KP. Dystroglycan inside and out. Curr Opin Cell Biol 1999;11:602-607.
155. Brancaccio A, Schulthess T, Gesemann M, Engel J. The N-terminal region of alpha-dystroglycan is an autonomous globular domain. Eur J Biochem 1997;246:166-172.
156. Brancaccio A, Ruegg MA, Engel J. Cloning and sequencing of mouse skeletal muscle alpha-dystroglycan. Matrix Biol 1995;14:681-685.
157. Katoh K, Omori Y, Furukawa T. [Extracellular matrix protein, Pikachurin, is required for photoreceptor ribbon synapse formation]. Tanpakushitsu Kakusan Koso 2009;54:1166-1172.
158. Barresi R, Campbell KP. Dystroglycan: from biosynthesis to pathogenesis of human disease. J Cell Sci 2006;119:199-207.
159. Cao W, Henry MD, Borrow P, et al. Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus. Science 1998;282:2079-2081.
160. Rambukkana A, Yamada H, Zanazzi G, et al. Role of alpha-dystroglycan as a Schwann cell receptor for Mycobacterium leprae. Science 1998;282:2076-2079.
161. Williamson RA, Henry MD, Daniels KJ, et al. Dystroglycan is essential for early embryonic development: disruption of Reichert's membrane in Dag1-null mice. Hum Mol Genet 1997;6:831-841.
162. Henry MD, Campbell KP. A role for dystroglycan in basement membrane assembly. Cell 1998;95:859-870.
163. Michele DE, Barresi R, Kanagawa M, et al. Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. Nature 2002;418:417-422.
164. Cote PD, Moukhles H, Lindenbaum M, Carbonetto S. Chimaeric mice deficient in dystroglycans develop muscular dystrophy and have disrupted myoneural synapses. Nat Genet 1999;23:338-342.
165. Cohn RD, Henry MD, Michele DE, et al. Disruption of DAG1 in differentiated skeletal muscle reveals a role for dystroglycan in muscle regeneration. Cell 2002;110:639-648.
166. Ayalon G, Davis JQ, Scotland PB, Bennett V. An ankyrin-based mechanism for functional organization of dystrophin and dystroglycan. Cell 2008;135:1189-1200.
167. Cartaud A, Coutant S, Petrucci TC, Cartaud J. Evidence for in situ and in vitro association between beta-dystroglycan and the subsynaptic 43K rapsyn protein.

Consequence for acetylcholine receptor clustering at the synapse. J Biol Chem 1998;273:11321-11326.
168. Jacobson C, Cote PD, Rossi SG, Rotundo RL, Carbonetto S. The dystroglycan complex is necessary for stabilization of acetylcholine receptor clusters at neuromuscular junctions and formation of the synaptic basement membrane. J Cell Biol 2001;152:435-450.
169. Moukhles H, Roque R, Carbonetto S. alpha-dystroglycan isoforms are differentially distributed in adult rat retina. J Comp Neurol 2000;420:182-194.
170. Kanagawa M, Omori Y, Sato S, et al. Post-translational maturation of dystroglycan is necessary for pikachurin binding and ribbon synaptic localization. J Biol Chem;285:31208-31216.
171. Montanaro F, Carbonetto S. Targeting dystroglycan in the brain. Neuron 2003;37:193-196.
172. Durbeej M, Larsson E, Ibraghimov-Beskrovnaya O, Roberds SL, Campbell KP, Ekblom P. Non-muscle alpha-dystroglycan is involved in epithelial development. J Cell Biol 1995;130:79-91.
173. Durbeej M, Talts JF, Henry MD, Yurchenco PD, Campbell KP, Ekblom P. Dystroglycan binding to laminin alpha1LG4 module influences epithelial morphogenesis of salivary gland and lung in vitro. Differentiation 2001;69:121-134.
174. Hayashi YK, Engvall E, Arikawa-Hirasawa E, et al. Abnormal localization of laminin subunits in muscular dystrophies. J Neurol Sci 1993;119:53-64.
175. Saito Y, Murayama S, Kawai M, Nakano I. Breached cerebral glia limitans-basal lamina complex in Fukuyama-type congenital muscular dystrophy. Acta Neuropathol 1999;98:330-336.
176. Hayashi YK, Ogawa M, Tagawa K, et al. Selective deficiency of alpha-dystroglycan in Fukuyama-type congenital muscular dystrophy. Neurology 2001;57:115-121.
177. Grewal PK, Holzfeind PJ, Bittner RE, Hewitt JE. Mutant glycosyltransferase and altered glycosylation of alpha-dystroglycan in the myodystrophy mouse. Nat Genet 2001;28:151-154.
178. Holzfeind PJ, Grewal PK, Reitsamer HA, et al. Skeletal, cardiac and tongue muscle pathology, defective retinal transmission, and neuronal migration defects in the Large(myd) mouse defines a natural model for glycosylation-deficient muscle - eye - brain disorders. Hum Mol Genet 2002;11:2673-2687.
179. Kobata A. Structures and functions of the sugar chains of glycoproteins. Eur J Biochem 1992;209:483-501.
180. Varki A. Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 1993;3:97-130.
181. Freeze HH. Genetic defects in the human glycome. Nat Rev Genet 2006;7:537-551.
182. Grunewald S. Congenital disorders of glycosylation: rapidly enlarging group of (neuro)metabolic disorders. Early Hum Dev 2007;83:825-830.
183. Endo T. O-mannosyl glycans in mammals. Biochim Biophys Acta 1999;1473:237-246.
184. Yamada H, Chiba A, Endo T, et al. Characterization of dystroglycan-laminin interaction in peripheral nerve. J Neurochem 1996;66:1518-1524.
185. Martin PT. Dystroglycan glycosylation and its role in matrix binding in skeletal muscle. Glycobiology 2003;13:55R-66R.
186. Hoyte K, Kang C, Martin PT. Definition of pre- and postsynaptic forms of the CT carbohydrate antigen at the neuromuscular junction: ubiquitous expression of the

CT antigens and the CT GalNAc transferase in mouse tissues. Brain Res Mol Brain Res 2002;109:146-160.
187. Smalheiser NR, Kim E. Purification of cranin, a laminin binding membrane protein. Identity with dystroglycan and reassessment of its carbohydrate moieties. J Biol Chem 1995;270:15425-15433.
188. Chiba A, Matsumura K, Yamada H, et al. Structures of sialylated O-linked oligosaccharides of bovine peripheral nerve alpha-dystroglycan. The role of a novel O-mannosyl-type oligosaccharide in the binding of alpha-dystroglycan with laminin. J Biol Chem 1997;272:2156-2162.
189. Jurado LA, Coloma A, Cruces J. Identification of a human homolog of the Drosophila rotated abdomen gene (POMT1) encoding a putative protein O-mannosyl-transferase, and assignment to human chromosome 9q34.1. Genomics 1999;58:171-180.
190. Manya H, Chiba A, Yoshida A, et al. Demonstration of mammalian protein O-mannosyltransferase activity: coexpression of POMT1 and POMT2 required for enzymatic activity. Proc Natl Acad Sci U S A 2004;101:500-505.
191. Willer T, Amselgruber W, Deutzmann R, Strahl S. Characterization of POMT2, a novel member of the PMT protein O-mannosyltransferase family specifically localized to the acrosome of mammalian spermatids. Glycobiology 2002;12:771-783.
192. Brockington M, Yuva Y, Prandini P, et al. Mutations in the fukutin-related protein gene (FKRP) identify limb girdle muscular dystrophy 2I as a milder allelic variant of congenital muscular dystrophy MDC1C. Hum Mol Genet 2001;10:2851-2859.
193. Matsumoto H, Noguchi S, Sugie K, et al. Subcellular localization of fukutin and fukutin-related protein in muscle cells. J Biochem 2004;135:709-712.
194. Keramaris-Vrantsis E, Lu PJ, Doran T, et al. Fukutin-related protein localizes to the Golgi apparatus and mutations lead to mislocalization in muscle in vivo. Muscle Nerve 2007;36:455-465.
195. Esapa CT, Benson MA, Schroder JE, et al. Functional requirements for fukutin-related protein in the Golgi apparatus. Hum Mol Genet 2002;11:3319-3331.
196. Beedle AM, Nienaber PM, Campbell KP. Fukutin-related protein associates with the sarcolemmal dystrophin-glycoprotein complex. J Biol Chem 2007;282:16713-16717.
197. Peyrard M, Seroussi E, Sandberg-Nordqvist AC, et al. The human LARGE gene from 22q12.3-q13.1 is a new, distinct member of the glycosyltransferase gene family. Proc Natl Acad Sci U S A 1999;96:598-603.
198. Grewal PK, McLaughlan JM, Moore CJ, Browning CA, Hewitt JE. Characterization of the LARGE family of putative glycosyltransferases associated with dystroglycanopathies. Glycobiology 2005;15:912-923.
199. Kanagawa M, Saito F, Kunz S, et al. Molecular recognition by LARGE is essential for expression of functional dystroglycan. Cell 2004;117:953-964.
200. Brockington M, Torelli S, Prandini P, et al. Localization and functional analysis of the LARGE family of glycosyltransferases: significance for muscular dystrophy. Hum Mol Genet 2005;14:657-665.
201. Muntoni F, Torelli S, Brockington M. Muscular dystrophies due to glycosylation defects. Neurotherapeutics 2008;5:627-632.
202. Yoshida-Moriguchi T, Yu L, Stalnaker SH, et al. O-mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding. Science;327:88-92.
203. Henry MD, Cohen MB, Campbell KP. Reduced expression of dystroglycan in breast and prostate cancer. Hum Pathol 2001;32:791-795.

204. Singh J, Itahana Y, Knight-Krajewski S, et al. Proteolytic enzymes and altered glycosylation modulate dystroglycan function in carcinoma cells. Cancer Res 2004;64:6152-6159.
205. Mercuri E, Brockington M, Straub V, et al. Phenotypic spectrum associated with mutations in the fukutin-related protein gene. Ann Neurol 2003;53:537-542.
206. Cormand B, Pihko H, Bayes M, et al. Clinical and genetic distinction between Walker-Warburg syndrome and muscle-eye-brain disease. Neurology 2001;56:1059-1069.
207. Beltran-Valero de Bernabe D, Voit T, Longman C, et al. Mutations in the FKRP gene can cause muscle-eye-brain disease and Walker-Warburg syndrome. J Med Genet 2004;41:e61.
208. de Bernabe DB, van Bokhoven H, van Beusekom E, et al. A homozygous nonsense mutation in the fukutin gene causes a Walker-Warburg syndrome phenotype. J Med Genet 2003;40:845-848.
209. Cormand B, Avela K, Pihko H, et al. Assignment of the muscle-eye-brain disease gene to 1p32-p34 by linkage analysis and homozygosity mapping. Am J Hum Genet 1999;64:126-135.
210. Mercuri E, D'Amico A, Tessa A, et al. POMT2 mutation in a patient with 'MEB-like' phenotype. Neuromuscul Disord 2006;16:446-448.
211. Topaloglu H, Brockington M, Yuva Y, et al. FKRP gene mutations cause congenital muscular dystrophy, mental retardation, and cerebellar cysts. Neurology 2003;60:988-992.
212. Fukuyama Y KM, Haruna H. A perculiar form of congenital progressive muscular dystrophy:report of fifteen cases. Paediat Univ Tokyo 1960;4:5-8.
213. Clement E, Mercuri E, Godfrey C, et al. Brain involvement in muscular dystrophies with defective dystroglycan glycosylation. Ann Neurol 2008;64:573-582.
214. Toda T, Kobayashi K, Kondo-Iida E, Sasaki J, Nakamura Y. The Fukuyama congenital muscular dystrophy story. Neuromuscul Disord 2000;10:153-159.
215. Toda T, Segawa M, Nomura Y, et al. Localization of a gene for Fukuyama type congenital muscular dystrophy to chromosome 9q31-33. Nat Genet 1993;5:283-286.
216. Mercuri E, Sewry CA, Brown SC, et al. Congenital muscular dystrophy with secondary merosin deficiency and normal brain MRI: a novel entity? Neuropediatrics 2000;31:186-189.
217. Dincer P, Balci B, Yuva Y, et al. A novel form of recessive limb girdle muscular dystrophy with mental retardation and abnormal expression of alpha-dystroglycan. Neuromuscul Disord 2003;13:771-778.
218. Jimenez-Mallebrera C, Torelli S, Feng L, et al. A comparative study of alpha-dystroglycan glycosylation in dystroglycanopathies suggests that the hypoglycosylation of alpha-dystroglycan does not consistently correlate with clinical severity. Brain Pathol 2009;19:596-611.
219. Zhang W, Vajsar J, Cao P, et al. Enzymatic diagnostic test for Muscle-Eye-Brain type congenital muscular dystrophy using commercially available reagents. Clin Biochem 2003;36:339-344.
220. Vajsar J, Zhang W, Dobyns WB, et al. Carriers and patients with muscle-eye-brain disease can be rapidly diagnosed by enzymatic analysis of fibroblasts and lymphoblasts. Neuromuscul Disord 2006;16:132-136.
221. Mercuri E, Longman C. Congenital muscular dystrophy. Pediatr Ann 2005;34:560-562, 564-568.
222. Klein A, Clement E, Mercuri E, Muntoni F. Differential diagnosis of congenital muscular dystrophies. Eur J Paediatr Neurol 2008;12:371-377.

223. Darin N, Kimber E, Kroksmark AK, Tulinius M. Multiple congenital contractures: birth prevalence, etiology, and outcome. J Pediatr 2002;140:61-67.
224. Osawa M SS, Suzuki N, Arai Y, Ikenaka H, Murasugi H, Shishikura K, Suzuki H, Saito K, Fukuyama Y. . Fukuyama type congenital muscular dystrophy. In: Congenital Muscular Dystrophies: Elsevier Science, 1997.
225. Toda T. [Fukuyama-type congenital muscular dystrophy]. Rinsho Shinkeigaku 2000;40:1297-1299.
226. Silan F, Yoshioka M, Kobayashi K, et al. A new mutation of the fukutin gene in a non-Japanese patient. Ann Neurol 2003;53:392-396.
227. Vuillaumier-Barrot S, Quijano-Roy S, Bouchet-Seraphin C, et al. Four Caucasian patients with mutations in the fukutin gene and variable clinical phenotype. Neuromuscul Disord 2009;19:182-188.
228. Okada M, Kawahara G, Noguchi S, et al. Primary collagen VI deficiency is the second most common congenital muscular dystrophy in Japan. Neurology 2007;69:1035-1042.
229. Peat RA, Smith JM, Compton AG, et al. Diagnosis and etiology of congenital muscular dystrophy. Neurology 2008;71:312-321.
230. Norwood FL, Harling C, Chinnery PF, Eagle M, Bushby K, Straub V. Prevalence of genetic muscle disease in Northern England: in-depth analysis of a muscle clinic population. Brain 2009;132:3175-3186.
231. Godfrey C, Clement E, Mein R, et al. Refining genotype phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan. Brain 2007;130:2725-2735.
232. Nadeau A, Kinali M, Main M, et al. Natural history of Ullrich congenital muscular dystrophy. Neurology 2009;73:25-31.
233. Kinali M, Beeson D, Pitt MC, et al. Congenital myasthenic syndromes in childhood: diagnostic and management challenges. J Neuroimmunol 2008;201-202:6-12.
234. Schessl J, Goemans NM, Magold AI, et al. Predominant fiber atrophy and fiber type disproportion in early ullrich disease. Muscle Nerve 2008;38:1184-1191.
235. Warburg M. Hydrocephaly, congenital retinal nonattachment, and congenital falciform fold. Am J Ophthalmol 1978;85:88-94.
236. Walker AE. Lissencephaly. Arch Neurol Psychiat 1942;48:13-29.
237. Whitley CB, Thompson TT, Mastri AR, Gorlin RJ. Warburg syndrome: lethal neurodysplasia with autosomal recessive inheritance. J Pediatr 1983;102:547-552.
238. Williams RS, Swisher CN, Jennings M, Ambler M, Caviness VS, Jr. Cerebro-ocular dysgenesis (Walker-Warburg syndrome): neuropathologic and etiologic analysis. Neurology 1984;34:1531-1541.
239. Pagon RA, Chandler JW, Collie WR, et al. Hydrocephalus, agyria, retinal dysplasia, encephalocele (HARD +/- E) syndrome: an autosomal recessive condition. Birth Defects Orig Artic Ser 1978;14:233-241.
240. Towfighi J, Sassani JW, Suzuki K, Ladda RL. Cerebro-ocular dysplasia-muscular dystrophy (COD-MD) syndrome. Acta Neuropathol 1984;65:110-123.
241. Dobyns WB, Pagon RA, Armstrong D, et al. Diagnostic criteria for Walker-Warburg syndrome. Am J Med Genet 1989;32:195-210.
242. Santavuori P, Leisti J, Kruus S. Muscle, eye and brain disease: a new syndrome. Neuropediatrie 1977;8 (suppl):553-558.
243. Santavuori P, Pihko H, Sainio K, et al. Muscle-eye-brain disease and Walker-Warburg syndrome. Am J Med Genet 1990;36:371-374.
244. Santavuori P, Somer H, Sainio K, et al. Muscle-eye-brain disease (MEB). Brain Dev 1989;11:147-153.

245. Yoshioka M, Toda T, Kuroki S, Hamano K. Broader clinical spectrum of Fukuyama-type congenital muscular dystrophy manifested by haplotype analysis. J Child Neurol 1999;14:711-715.
246. Fukuyama Y, Osawa M, Suzuki H. Congenital progressive muscular dystrophy of the Fukuyama type - clinical, genetic and pathological considerations. Brain Dev 1981;3:1-29.
247. Olson EC, Walsh CA. Smooth, rough and upside-down neocortical development. Curr Opin Genet Dev 2002;12:320-327.
248. Santavuori P, Pihko H, Sainio K, et al. Muscle-eye-brain disease and Walker-Warburg syndrome. (letter). Am J Med Genet 1990;36:371-372.
249. Dobyns W, Pagon RA, Curry CJ, Greenberg F. Response to Santavuori et al. regarding Walker -Warburg syndrome and muscle-eye-brain disease. (letler). Am J Med Genet 1990;36:373-374.
250. Yoshioka M, Kuroki S. Clinical spectrum and genetic studies of Fukuyama congenital muscular dystrophy. Am J Med Genet 1994;53:245-250.
251. van Reeuwijk J, Maugenre S, van den Elzen C, et al. The expanding phenotype of POMT1 mutations: from Walker-Warburg syndrome to congenital muscular dystrophy, microcephaly, and mental retardation. Hum Mutat 2006;27:453-459.
252. D'Amico A, Tessa A, Bruno C, et al. Expanding the clinical spectrum of POMT1 phenotype. Neurology 2006;66:1564-1567; discussion 1461.
253. Currier SC, Lee CK, Chang BS, et al. Mutations in POMT1 are found in a minority of patients with Walker-Warburg syndrome. Am J Med Genet A 2005;133A:53-57.
254. Taniguchi K, Kobayashi K, Saito K, et al. Worldwide distribution and broader clinical spectrum of muscle-eye-brain disease. Hum Mol Genet 2003;12:527-534.
255. Kondo-Iida E, Kobayashi K, Watanabe M, et al. Novel mutations and genotype-phenotype relationships in 107 families with Fukuyama-type congenital muscular dystrophy (FCMD). Hum Mol Genet 1999;8:2303-2309.
256. Brown SC, Torelli S, Brockington M, et al. Abnormalities in alpha-dystroglycan expression in MDC1C and LGMD2I muscular dystrophies. Am J Pathol 2004;164:727-737.
257. Godfrey C, Escolar D, Brockington M, et al. Fukutin gene mutations in steroid-responsive limb girdle muscular dystrophy. Ann Neurol 2006;60:603-610.
258. Mercuri E, Topaloglu H, Brockington M, et al. Spectrum of brain changes in patients with congenital muscular dystrophy and FKRP gene mutations. Arch Neurol 2006;63:251-257.
259. Diesen C, Saarinen A, Pihko H, et al. POMGnT1 mutation and phenotypic spectrum in muscle-eye-brain disease. J Med Genet 2004;41:e115.
260. Clement EMG, C.; Tan, J.; et.al.. Mild POMGnT1 mutations underlie a novel Limb Girdle Muscular Dystrophy varian. Arch Neurol 2007.
261. van Reeuwijk J, Grewal PK, Salih MA, et al. Intragenic deletion in the LARGE gene causes Walker-Warburg syndrome. Hum Genet 2007.
262. Matsumoto H, Hayashi YK, Kim DS, et al. Congenital muscular dystrophy with glycosylation defects of alpha-dystroglycan in Japan. Neuromuscul Disord 2005;15:342-348.
263. Torelli S, Brown SC, Brockington M, et al. Sub-cellular localisation of fukutin related protein in different cell lines and in the muscle of patients with MDC1C and LGMD2I. Neuromuscul Disord 2005;15:836-843.
264. Quijano-Roy S, Marti-Carrera I, Makri S, et al. Brain MRI abnormalities in muscular dystrophy due to FKRP mutations. Brain Dev 2006;28:232-242.

265. Harel T, Goldberg Y, Shalev SA, Chervinski I, Ofir R, Birk OS. Limb-girdle muscular dystrophy 2I: phenotypic variability within a large consanguineous Bedouin family associated with a novel FKRP mutation. Eur J Hum Genet 2004;12:38-43.
266. Vieira NM, Schlesinger D, de Paula F, Vainzof M, Zatz M. Mutation analysis in the FKRP gene provides an explanation for a rare cause of intrafamilial clinical variability in LGMD2I. Neuromuscul Disord 2006;16:870-873.
267. de Paula F, Vieira N, Starling A, et al. Asymptomatic carriers for homozygous novel mutations in the FKRP gene: the other end of the spectrum. Eur J Hum Genet 2003;11:923-930.
268. Lin YC, Murakami T, Hayashi YK, et al. A novel FKRP gene mutation in a Taiwanese patient with limb-girdle muscular dystrophy 2I. Brain Dev 2007;29:234-238.
269. Boito CA, Melacini P, Vianello A, et al. Clinical and molecular characterization of patients with limb-girdle muscular dystrophy type 2I. Arch Neurol 2005;62:1894-1899.
270. Frosk P, Greenberg CR, Tennese AA, et al. The most common mutation in FKRP causing limb girdle muscular dystrophy type 2I (LGMD2I) may have occurred only once and is present in Hutterites and other populations. Hum Mutat 2005;25:38-44.
271. Bourteel H, Stojkovic T, Cuisset JM, et al. [Phenotypic aspects of FKRP-linked muscular dystrophy type 2I in a series of eleven patients.]. Rev Neurol (Paris) 2007;163:189-196.
272. Zhang W, Betel D, Schachter H. Cloning and expression of a novel UDP-GlcNAc:alpha-D-mannoside beta1,2-N-acetylglucosaminyltransferase homologous to UDP-GlcNAc:alpha-3-D-mannoside beta1,2-N-acetylglucosaminyltransferase I. Biochem J 2002;361:153-162.
273. Lumsden A, Krumlauf R. Patterning the vertebrate neuraxis. Science 1996;274:1109-1115.
274. Jansen A, Andermann E. Genetics of the polymicrogyria syndromes. J Med Genet 2005;42:369-378.
275. Barkovich AJ, Kuzniecky RI, Jackson GD, Guerrini R, Dobyns WB. Classification system for malformations of cortical development: update 2001. Neurology 2001;57:2168-2178.
276. Guerrini R, Marini C. Genetic malformations of cortical development. Exp Brain Res 2006;173:322-333.
277. Robain O. Introduction to the pathology of cerebral cortical dysplasia. In: Guerrini R, Andermann F, Canapicchi R, Roger J, Zifkin B, Pfanner P, eds. Dysplasias of cerebral cortex and epilepsy. Philidelphia: Lippincott-Raven, 1996: 1-9.
278. Gleeson JG, Walsh CA. Neuronal migration disorders: from genetic diseases to developmental mechanisms. Trends Neurosci 2000;23:352-359.
279. Halfter W, Dong S, Yip YP, Willem M, Mayer U. A critical function of the pial basement membrane in cortical histogenesis. J Neurosci 2002;22:6029-6040.
280. Graus-Porta D, Blaess S, Senften M, et al. Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. Neuron 2001;31:367-379.
281. Marret S, Mukendi R, Gadisseux JF, Gressens P, Evrard P. Effect of ibotenate on brain development: an excitotoxic mouse model of microgyria and posthypoxic-like lesions. J Neuropathol Exp Neurol 1995;54:358-370.
282. Piao X, Hill RS, Bodell A, et al. G protein-coupled receptor-dependent development of human frontal cortex. Science 2004;303:2033-2036.
283. Barkovich AJ, Rowley H, Bollen A. Correlation of prenatal events with the development of polymicrogyria. AJNR Am J Neuroradiol 1995;16:822-827.

284. Barkovich AJ. Neuroimaging manifestations and classification of congenital muscular dystrophies. AJNR Am J Neuroradiol 1998;19:1389-1396.
285. Takanashi J, Barkovich AJ. The changing MR imaging appearance of polymicrogyria: a consequence of myelination. AJNR Am J Neuroradiol 2003;24:788-793.
286. Barkovich AJ, Millen KJ, Dobyns WB. A developmental and genetic classification for midbrain-hindbrain malformations. Brain 2009;132:3199-3230.
287. Henion TR, Qu Q, Smith FI. Expression of dystroglycan, fukutin and POMGnT1 during mouse cerebellar development. Brain Res Mol Brain Res 2003;112:177-181.
288. Qu Q, Crandall JE, Luo T, McCaffery PJ, Smith FI. Defects in tangential neuronal migration of pontine nuclei neurons in the Largemyd mouse are associated with stalled migration in the ventrolateral hindbrain. Eur J Neurosci 2006;23:2877-2886.
289. Zaccaria ML, Di Tommaso F, Brancaccio A, Paggi P, Petrucci TC. Dystroglycan distribution in adult mouse brain: a light and electron microscopy study. Neuroscience 2001;104:311-324.
290. Qu Q, Smith FI. Alpha-dystroglycan interactions affect cerebellar granule neuron migration. J Neurosci Res 2004;76:771-782.
291. Hu H, Yang Y, Eade A, Xiong Y, Qi Y. Breaches of the pial basement membrane and disappearance of the glia limitans during development underlie the cortical lamination defect in the mouse model of muscle-eye-brain disease. J Comp Neurol 2007;501:168-183.
292. Kurahashi H, Taniguchi M, Meno C, et al. Basement membrane fragility underlies embryonic lethality in fukutin-null mice. Neurobiol Dis 2005;19:208-217.
293. Willer T, Prados B, Falcon-Perez JM, et al. Targeted disruption of the Walker-Warburg syndrome gene Pomt1 in mouse results in embryonic lethality. Proc Natl Acad Sci U S A 2004;101:14126-14131.
294. Chiyonobu T, Sasaki J, Nagai Y, et al. Effects of fukutin deficiency in the developing mouse brain. Neuromuscul Disord 2005;15:416-426.
295. Barkovich AJ, Millen KJ, Dobyns WB. A developmental classification of malformations of the brainstem. Ann Neurol 2007.
296. Jissendi-Tchofo P, Kara S, Barkovich AJ. Midbrain-hindbrain involvement in lissencephalies. Neurology 2009;72:410-418.
297. Haltia M, Leivo I, Somer H, et al. Muscle-eye-brain disease: a neuropathological study. Ann Neurol 1997;41:173-180.
298. Valanne L, Pihko H, Katevuo K, Karttunen P, Somer H, Santavuori P. MRI of the brain in muscle-eye-brain (MEB) disease. Neuroradiology 1994;36:473-476.
299. Aida N, Yagishita A, Takada K, Katsumata Y. Cerebellar MR in Fukuyama congenital muscular dystrophy: polymicrogyria with cystic lesions. AJNR Am J Neuroradiol 1994;15:1755-1759.
300. Takada K, Nakamura H, Tanaka J. Cortical dysplasia in congenital muscular dystrophy with central nervous system involvement (Fukuyama type). J Neuropathol Exp Neurol 1984;43:395-407.
301. Chang BS, Piao X, Bodell A, et al. Bilateral frontoparietal polymicrogyria: clinical and radiological features in 10 families with linkage to chromosome 16. Ann Neurol 2003;53:596-606.
302. Piao X, Chang BS, Bodell A, et al. Genotype-phenotype analysis of human frontoparietal polymicrogyria syndromes. Ann Neurol 2005;58:680-687.
303. Li S, Jin Z, Koirala S, et al. GPR56 regulates pial basement membrane integrity and cortical lamination. J Neurosci 2008;28:5817-5826.

304. Koirala S, Jin Z, Piao X, Corfas G. GPR56-regulated granule cell adhesion is essential for rostral cerebellar development. J Neurosci 2009;29:7439-7449.
305. Kornak U, Reynders E, Dimopoulou A, et al. Impaired glycosylation and cutis laxa caused by mutations in the vesicular H+-ATPase subunit ATP6V0A2. Nat Genet 2008;40:32-34.
306. Van Maldergem L, Yuksel-Apak M, Kayserili H, et al. Cobblestone-like brain dysgenesis and altered glycosylation in congenital cutis laxa, Debre type. Neurology 2008;71:1602-1608.
307. Sprecher E, Ishida-Yamamoto A, Mizrahi-Koren M, et al. A mutation in SNAP29, coding for a SNARE protein involved in intracellular trafficking, causes a novel neurocutaneous syndrome characterized by cerebral dysgenesis, neuropathy, ichthyosis, and palmoplantar keratoderma. Am J Hum Genet 2005;77:242-251.
308. Louhichi N, Triki C, Quijano-Roy S, et al. New FKRP mutations causing congenital muscular dystrophy associated with mental retardation and central nervous system abnormalities. Identification of a founder mutation in Tunisian families. Neurogenetics 2004;5:27-34.
309. Yanagisawa A, Bouchet C, Van den Bergh PY, et al. New POMT2 mutations causing congenital muscular dystrophy. Neurology 2007.
310. Biancheri R, Bertini E, Falace A, et al. POMGnT1 mutations in congenital muscular dystrophy: genotype-phenotype correlation and expanded clinical spectrum. Arch Neurol 2006;63:1491-1495.
311. van Reeuwijk J, Grewal PK, Salih MA, et al. Intragenic deletion in the LARGE gene causes Walker-Warburg syndrome. Hum Genet 2007;121:685-690.
312. Liu J, Ball SL, Yang Y, et al. A genetic model for muscle-eye-brain disease in mice lacking protein O-mannose 1,2-N-acetylglucosaminyltransferase (POMGnT1). Mech Dev 2006;123:228-240.
313. Friede R. Developmental Neuropathology. Berlin: Springer-Verlag, 1989.
314. Forman MS, Squier W, Dobyns WB, Golden JA. Genotypically defined lissencephalies show distinct pathologies. J Neuropathol Exp Neurol 2005;64:847-857.
315. Takada K, Nakamura H, Takashima S. Cortical dysplasia in Fukuyama congenital muscular dystrophy (FCMD): a Golgi and angioarchitectonic analysis. Acta Neuropathol 1988;76:170-178.
316. Barkovich AJ, Koch TK, Carrol CL. The spectrum of lissencephaly: report of ten patients analyzed by magnetic resonance imaging. Ann Neurol 1991;30:139-146.
317. Mercuri E, Dubowitz L, Brown SP, Cowan F. Incidence of cranial ultrasound abnormalities in apparently well neonates on a postnatal ward: correlation with antenatal and perinatal factors and neurological status. Arch Dis Child Fetal Neonatal Ed 1998;79:F185-189.
318. Aida N, Tamagawa K, Takada K, et al. Brain MR in Fukuyama congenital muscular dystrophy. AJNR Am J Neuroradiol 1996;17:605-613.
319. Vervoort VS, Holden KR, Ukadike KC, Collins JS, Saul RA, Srivastava AK. POMGnT1 gene alterations in a family with neurological abnormalities. Ann Neurol 2004;56:143-148.
320. Sunada Y, Edgar TS, Lotz BP, Rust RS, Campbell KP. Merosin-negative congenital muscular dystrophy associated with extensive brain abnormalities. Neurology 1995;45:2084-2089.
321. Muntoni F, Guicheney P, Voit T. 158th ENMC international workshop on congenital muscular dystrophy (Xth international CMD workshop) 8th-10th February 2008 Naarden, The Netherlands. Neuromuscul Disord 2009;19:229-234.
322. Gros-Louis F, Dupre N, Dion P, et al. Mutations in SYNE1 lead to a newly discovered form of autosomal recessive cerebellar ataxia. Nat Genet 2007;39:80-85.

323. Apel ED, Lewis RM, Grady RM, Sanes JR. Syne-1, a dystrophin- and Klarsicht-related protein associated with synaptic nuclei at the neuromuscular junction. J Biol Chem 2000;275:31986-31995.
324. Mislow JM, Holaska JM, Kim MS, et al. Nesprin-1alpha self-associates and binds directly to emerin and lamin A in vitro. FEBS Lett 2002;525:135-140.
325. Hansske B, Thiel C, Lubke T, et al. Deficiency of UDP-galactose:N-acetylglucosamine beta-1,4-galactosyltransferase I causes the congenital disorder of glycosylation type IId. J Clin Invest 2002;109:725-733.
326. Peters V, Penzien JM, Reiter G, et al. Congenital disorder of glycosylation IId (CDG-IId) -- a new entity: clinical presentation with Dandy-Walker malformation and myopathy. Neuropediatrics 2002;33:27-32.
327. Mercuri E, Messina S, Bruno C, et al. Congenital muscular dystrophies with defective glycosylation of dystroglycan: a population study. Neurology 2009;72:1802-1809.
328. Bouchet C, Gonzales M, Vuillaumier-Barrot S, et al. Molecular heterogeneity in fetal forms of type II lissencephaly. Hum Mutat 2007;28:1020-1027.
329. Manzini MC, Gleason D, Chang BS, et al. Ethnically diverse causes of Walker-Warburg syndrome (WWS): FCMD mutations are a more common cause of WWS outside of the Middle East. Hum Mutat 2008;29:E231-241.
330. Puckett RL, Moore SA, Winder TL, et al. Further evidence of Fukutin mutations as a cause of childhood onset limb-girdle muscular dystrophy without mental retardation. Neuromuscul Disord 2009;19:352-356.
331. Yanagisawa A, Bouchet C, Quijano-Roy S, et al. POMT2 intragenic deletions and splicing abnormalities causing congenital muscular dystrophy with mental retardation. Eur J Med Genet 2009;52:201-206.
332. Yanagisawa A, Bouchet C, Van den Bergh PY, et al. New POMT2 mutations causing congenital muscular dystrophy: identification of a founder mutation. Neurology 2007;69:1254-1260.
333. Godfrey CC, E.; Mein, R.; Brockington, M.; Smith, J.; Talim, B.; Straub, V.; Robb, S.; Quinlivan R.; Feng, L.; Jimenez-Mallebrera, C.; Mercuri, E.; Manzur, A.; Kinali, M.; Torelli, S.; Brown, S.; Sewry, C.; Bushby, K.; Topaloglu, H.; North, K.; Abbs, S.; Muntoni, F. Refining genotype-phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan. Brain 2007.
334. Biancheri R, Falace A, Tessa A, et al. POMT2 gene mutation in limb-girdle muscular dystrophy with inflammatory changes. Biochem Biophys Res Commun 2007;363:1033-1037.
335. Chung W, Winder TL, LeDuc CA, et al. Founder Fukutin mutation causes Walker-Warburg syndrome in four Ashkenazi Jewish families. Prenat Diagn 2009;29:560-569.
336. Jimenez-Mallebrera C, Torelli S, Feng L, et al. A Comparative Study of alpha-Dystroglycan Glycosylation in Dystroglycanopathies Suggests that the Hypoglycosylation of alpha-Dystroglycan Does Not Consistently Correlate with Clinical Severity. Brain Pathol 2008.
337. Ng SB, Buckingham KJ, Lee C, et al. Exome sequencing identifies the cause of a mendelian disorder. Nat Genet;42:30-35.
338. Ramelli GP, Aloysius A, King C, Davis T, Muntoni F. Gastrostomy placement in paediatric patients with neuromuscular disorders: indications and outcome. Dev Med Child Neurol 2007;49:367-371.
339. Merlini L, Bernardi P. Therapy of collagen VI-related myopathies (Bethlem and Ullrich). Neurotherapeutics 2008;5:613-618.

340. Takaso M, Nakazawa T, Imura T, et al. Surgical correction of spinal deformity in patients with congenital muscular dystrophy. J Orthop Sci;15:493-501.
341. Barresi R, Michele DE, Kanagawa M, et al. LARGE can functionally bypass alpha-dystroglycan glycosylation defects in distinct congenital muscular dystrophies. Nat Med 2004;10:696-703.
342. Smith PL, Lowe JB. Molecular cloning of a murine N-acetylgalactosamine transferase cDNA that determines expression of the T lymphocyte-specific CT oligosaccharide differentiation antigen. J Biol Chem 1994;269:15162-15171.
343. Xia B, Martin PT. Modulation of agrin binding and activity by the CT and related carbohydrate antigens. Mol Cell Neurosci 2002;19:539-551.
344. Blankinship MJ, Gregorevic P, Chamberlain JS. Gene therapy strategies for Duchenne muscular dystrophy utilizing recombinant adeno-associated virus vectors. Mol Ther 2006;13:241-249.
345. Angelin A, Tiepolo T, Sabatelli P, et al. Mitochondrial dysfunction in the pathogenesis of Ullrich congenital muscular dystrophy and prospective therapy with cyclosporins. Proc Natl Acad Sci U S A 2007;104:991-996.
346. Irwin WA, Bergamin N, Sabatelli P, et al. Mitochondrial dysfunction and apoptosis in myopathic mice with collagen VI deficiency. Nat Genet 2003;35:367-371.
347. Merlini L, Angelin A, Tiepolo T, et al. Cyclosporin A corrects mitochondrial dysfunction and muscle apoptosis in patients with collagen VI myopathies. Proc Natl Acad Sci U S A 2008;105:5225-5229.
348. Hansson MJ, Mattiasson G, Mansson R, et al. The nonimmunosuppressive cyclosporin analogs NIM811 and UNIL025 display nanomolar potencies on permeability transition in brain-derived mitochondria. J Bioenerg Biomembr 2004;36:407-413.
349. Tiepolo T, Angelin A, Palma E, et al. The cyclophilin inhibitor Debio 025 normalizes mitochondrial function, muscle apoptosis and ultrastructural defects in Col6a1-/- myopathic mice. Br J Pharmacol 2009;157:1045-1052.
350. Erb M, Meinen S, Barzaghi P, et al. Omigapil ameliorates the pathology of muscle dystrophy caused by laminin-alpha2 deficiency. J Pharmacol Exp Ther 2009;331:787-795.
351. Vachon PH, Loechel F, Xu H, Wewer UM, Engvall E. Merosin and laminin in myogenesis; specific requirement for merosin in myotube stability and survival. J Cell Biol 1996;134:1483-1497.
352. Hall TE, Bryson-Richardson RJ, Berger S, et al. The zebrafish candyfloss mutant implicates extracellular matrix adhesion failure in laminin alpha2-deficient congenital muscular dystrophy. Proc Natl Acad Sci U S A 2007;104:7092-7097.
353. Dominov JA, Kravetz AJ, Ardelt M, Kostek CA, Beermann ML, Miller JB. Muscle-specific BCL2 expression ameliorates muscle disease in laminin {alpha}2-deficient, but not in dystrophin-deficient, mice. Hum Mol Genet 2005;14:1029-1040.
354. Girgenrath M, Beermann ML, Vishnudas VK, Homma S, Miller JB. Pathology is alleviated by doxycycline in a laminin-alpha2-null model of congenital muscular dystrophy. Ann Neurol 2009;65:47-56.
355. Qiao C, Li J, Zhu T, et al. Amelioration of laminin-alpha2-deficient congenital muscular dystrophy by somatic gene transfer of miniagrin. Proc Natl Acad Sci U S A 2005;102:11999-12004.
356. Meinen S, Lin S, Ruegg MA. Treatment approaches in laminin α2 deficient congenital muscular dystrophy (MDC1A). . In: Neuromuscul Disord, 2009: EM.P.541.504.

357. Hagiwara H, Ohsawa Y, Asakura S, Murakami T, Teshima T, Sunada Y. Bone marrow transplantation improves outcome in a mouse model of congenital muscular dystrophy. FEBS Lett 2006;580:4463-4468.
358. Zissimopoulos S, Lai FA. Redox regulation of the ryanodine receptor/calcium release channel. Biochem Soc Trans 2006;34:919-921.
359. Ferreiro A, Monnier N, Romero NB, et al. A recessive form of central core disease, transiently presenting as multi-minicore disease, is associated with a homozygous mutation in the ryanodine receptor type 1 gene. Ann Neurol 2002;51:750-759.
360. Collins J, Bonnemann CG. Congenital muscular dystrophies: toward molecular therapeutic interventions. Curr Neurol Neurosci Rep;10:83-91.

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Refining genotype–phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan

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Muscular dystrophies with reduced glycosylation of α-dystroglycan (α-DG), commonly referred to as dystroglycanopathies, are a heterogeneous group of autosomal recessive conditions which include a wide spectrum of clinical severity. Reported phenotypes range from severe congenital onset Walker–Warburg syndrome (WWS) with severe structural brain and eye involvement, to relatively mild adult onset limb girdle muscular dystrophy (LGMD). Specific clinical syndromes were originally described in association with mutations in any one of six demonstrated or putative glycosyltransferases. Work performed on patients with mutations in the *FKRP* gene has identified that the spectrum of phenotypes due to mutations in this gene is much wider than originally assumed. To further define the mutation frequency and phenotypes associated with mutations in the other five genes, we studied a large cohort of patients with evidence of a dystroglycanopathy. Exclusion of mutations in *FKRP* was a prerequisite for participation in this study. Ninety-two probands were screened for mutations in *POMT1*, *POMT2*, *POMGnT1*, *fukutin* and *LARGE*. Homozygous and compound heterozygous mutations were detected in a total of 31 probands (34 individuals from 31 families); 37 different mutations were identified, of which 32 were novel. Mutations in *POMT2* were the most prevalent in our cohort with nine cases, followed by *POMT1* with eight cases, *POMGnT1* with seven cases, *fukutin* with six cases and *LARGE* with only a single case. All patients with *POMT1* and *POMT2* mutations had evidence of either structural or functional central nervous system involvement including four patients with mental retardation and a LGMD phenotype. In contrast mutations in *fukutin* and *POMGnT1* were detected in four patients with LGMD and no evidence of brain involvement. The majority of patients (six out of nine) with mutations in *POMT2* had a Muscle–Eye–Brain (MEB)-like condition. In addition we identified a mutation in the gene *LARGE* in a patient with WWS.
Our data expands the clinical phenotypes associated with *POMT1*, *POMT2*, *POMGnT1*, *fukutin* and *LARGE* mutations. Mutations in these five glycosyltransferase genes were detected in 34% of patients indicating that, after the exclusion of *FKRP*, the majority of patients with a dystroglycanopathy harbour mutations in novel genes.

Keywords: congenital muscular dystrophy; limb girdle muscular dystrophy; alpha dystroglycan; glycosylation; glycosyltransferase

Abbreviations: CMD = congenital muscular dystrophy, LGMD = limb girdle muscular dystrophy, α-DG = alpha-dystroglycan

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Introduction

Muscular dystrophies with reduced glycosylation of α-dystroglycan (α-DG) are a clinically and genetically heterogeneous group of autosomal recessive muscular dystrophies with variable neurological and ophthalmic involvement. Pathologically these disorders share the common feature of a hypoglycosylated form of α-dystroglycan (α-DG) on skeletal muscle biopsy (Muntoni *et al.*, 2002) which led to the term dystroglycanopathy (Toda *et al.*, 2003; Brockington and Muntoni, 2005; Mercuri *et al.*, 2006). Alpha and beta dystroglycan are derived from the same precursor peptide and are major components of the dystrophin-associated glycoprotein complex (DGC) that forms a link between the actin associated cytoskeleton and the extracellular matrix. α-DG is a highly glycosylated peripheral membrane protein that binds many of its extracellular matrix partners through its carbohydrate modifications. In the dystroglycanopathies, these modifications are either absent or reduced resulting in the decreased binding of its ligands, such as laminin-2, agrin and perlecan in skeletal muscle and neurexin in the brain (Barresi and Campbell, 2006). Primary mutations in the gene encoding dystroglycan (*DAG1*) have never been reported and a dystroglycan knockout mouse is embryonically lethal (Williamson *et al.*, 1997).

To date, mutations in six known or putative glycosyltransferase genes have been identified in these disorders: *Protein-O-mannosyl transferase* 1 (*POMT1*; OMIM 607423), *Protein-O-mannosyl transferase 2* (*POMT2*; OMIM 607439), *Protein-O-mannose 1,2-N-acetylglucosaminyltransferase 1* (*POMGnT1*; OMIM 606822), *fukutin* (OMIM 607440), *Fukutin-related protein* (*FKRP*; OMIM 606596) and *LARGE* (OMIM 603590) (Kobayashi *et al.*, 1998; Brockington *et al.*, 2001*a*; Yoshida *et al.*, 2001; Beltran-Valero de Bernabe *et al.*, 2002; Longman *et al.*, 2003; van Reeuwijk *et al.*, 2005*b*). These genes are thought to be involved in the addition of carbohydrate residues onto the α-DG backbone either via the process of *O*-mannosylation (*POMT1, POMT2, POMGnT1*) (Yoshida *et al.*, 2001; Manya *et al.*, 2003; Akasaka-Manya *et al.*, 2004) or via other not fully characterized mechanisms (*fukutin, FKRP and LARGE*) (de Paula *et al.*, 2003; Brown *et al.*, 2004; Brockington *et al.*, 2005; Xiong *et al.*, 2006).

The phenotypic severity of dystroglycanopathy patients is extremely variable. At the most severe end of the clinical spectrum are Walker–Warburg Syndrome (WWS), Muscle–Eye–Brain (MEB) disease and Fukuyama congenital muscular dystrophy (FCMD). These conditions are characterized by congenital muscular dystrophy (CMD) with severe structural brain and eye abnormalities, which in WWS results in early infantile death (van Reeuwijk *et al.*, 2005*a*). Conversely, individuals at the mildest end of the clinical spectrum may present, in adult life, with limb-girdle muscular dystrophy (LGMD) with no associated brain or eye involvement (Brockington *et al.*, 2001*b*). A number of intermediate phenotypes between these extremes have also been described including congenital muscular dystrophy type 1C (MDC1C), a CMD variant in which the brain can be entirely normal and LGMD2K, a variant with microcephaly and mental retardation but a relatively mild LGMD-like phenotype (Brockington *et al.*, 2001*a*; Balci *et al.*, 2005).

These syndromes were originally described in association with mutations in specific genes: WWS [OMIM 236670] was associated with mutations in *POMT1* and *POMT2* (Beltran-Valero de Bernabe *et al.*, 2002; Currier *et al.*, 2005; van Reeuwijk *et al.*, 2005*b*); these enzymes form a heterodimer and have been shown to catalyse the first step in *O*-mannosylation (Akasaka-Manya *et al.*, 2006). MEB [OMIM 253280] was originally described within the Finnish population in association with mutations in *POMGnT1*, an enzyme involved in the second step of *O*-mannosylation of α-DG by transferring *N*-acetylglucosamine to a protein *O*-linked mannose (Yoshida *et al.*, 2001). Recent molecular genetic studies have demonstrated that the high prevalence of MEB in the Finnish population is due to a founder splice site mutation (Diesen *et al.*, 2004). FCMD [OMIM 253800] was described within the Japanese population where it is the second most common form of muscular dystrophy after Duchenne muscular dystrophy (Kobayashi *et al.*, 1998). The high incidence of FCMD in Japan is related to a founder retrotransposal mutation in the 3′UTR of *fukutin*, which is found in the homozygous state in ~90% of all Japanese FCMD patients. MDC1C [OMIM 606612] and MDC1D [OMIM 608840] are two rare CMD syndromes, secondary to mutations in *FKRP and LARGE* respectively (Brockington *et al.*, 2001*a*; Longman *et al.*, 2003). The increased availability of mutation analysis in patients with a dystroglycanopathy has subsequently led to the widening of the clinical spectrum observed for several of these genes. This is best exemplified by the range of phenotypes resulting from mutations in *FKRP*. Following the initial description of its involvement in MDC1C (Brockington *et al.*, 2001*a*), it has subsequently been shown to cause a very common and relatively mild variant, LGMD2I [OMIM 607155] (Brockington *et al.*, 2001*b*), and more recently CMD variants with associated mild structural brain [MDC1C and cerebellar cysts (Topaloglu *et al.*, 2003; Mercuri *et al.*, 2006)] or severe brain and eye involvement (WWS and MEB-like disorders) (Beltran-Valero de Bernabe *et al.*, 2004; Mercuri *et al.*, 2006). It has recently been documented that several of these genes are involved in both milder and more severe phenotypes than originally reported. This includes the finding of *fukutin* mutations in two families with WWS (de Bernabe *et al.*, 2003) and in two families with a LGMD variant (Godfrey *et al.*, 2006) as well as the involvement of *POMT1* in patients with LGMD2K, with associated microcephaly and mental retardation (Balci *et al.*, 2005).

All previous studies have been conducted on a small number of families or individuals. This causes inevitable difficulties in applying mutation frequencies to the general population. In addition such reports make it difficult to establish whether the described clinical spectrum is truly representative of the phenotypic variability as well as how common the originally described core phenotypes are for each of these genes. In order to address these points, we have systematically screened a large population of patients with a dystroglycanopathy phenotype for mutations in the associated genes. As the spectrum of phenotypes secondary to *FKRP* involvement has been previously reported by us and others, we studied 92 patients in whom involvement of this gene had been excluded before proceeding with analysis of the five remaining genes. Our large and unbiased study redefines the clinical spectrum associated with each of the glycosyltransferases genes studied, identifies the frequency of individual gene defects and suggests that, after the exclusion of *FKRP*, the majority of patients with a dystroglycanopathy do not harbour mutations in any of the known genes.

Patient and methods

Patients

The cohort consisted of 92 unrelated individuals. This included a large group of patients from Australia (27 patients) and Turkey (16 patients). The majority of the remaining patients were recruited via the Hammersmith Hospital National Commissioning Group (NCG) service and included DNA from individuals referred from across the UK and Europe with a few samples from further a field. Mutations in *FKRP* had previously been excluded in all cases (Brockington *et al.*, 2001*a*).

The inclusion criteria were specified as hypoglycosylation of α-DG at the sarcolemma by immunolabelling of skeletal muscle sections (Brown *et al.*, 2004; Dubowitz and Sewry, 2007). Eighty patients met this criteria whilst in the remaining 12 cases there was no muscle available to perform α-DG studies. This later group of patients were included due to their clinical phenotype being highly suggestive of a dystroglycanopathy and consisted of children with CMD, elevated serum CK and brain MRI evocative of a cobblestone lissencephaly. All the patients who had had a muscle biopsy, were studied by standard immunocytochemical and/or Western blotting analysis in order to rule out dystrophinopathy, LGMDs such as sarcoglycanopathies, calpainopathy and dysferlinopathy, merosin deficient CMD and collagen VI deficiency (Dubowitz and Sewry, 2007). Clinical data was collated and patients were divided into phenotypic categories. This study was approved by Hammersmith Hospital Ethics Committee REC 2000:/5802.

Molecular genetics

Genomic DNA was extracted in the referring centre's laboratory using standard protocols. All mutation scanning was performed in the DNA laboratory at Guy's Hospital. The complete coding regions, including intron/exon boundaries of *POMT1*, *POMT2*, *POMGnT1*, *fukutin* and *LARGE* were amplified by PCR (primers are available in supplementary information, Table 1). Single nucleotide polymorphisms (SNP) within the primer binding sites were avoided using the Diagnostic SNP Check software (www.ngrl.man.ac.uk/SNPCheck). Amplicons were screened for mutations using a combination of unidirectional sequencing (standard dideoxynucleotide methodology) and heteroduplex analysis as previously described (Godfrey *et al.*, 2006). Where available, parental DNA was studied once a sequence alteration was identified in the proband. In two families, further segregation analysis was carried out to investigate the potential pathogenicity of unclassified variants. In families where a *de novo* mutation was suspected, paternity was confirmed using 11 STR markers (data not shown). Mutation nomenclature based on the following GeneBank Accession numbers; *POMT1*; NM_007171.2, *POMT2*; NM_013382.3, *POMGnT1*; NM_017739.1, *fukutin*; NM_006731.1 and *LARGE*; NM_133642.2, with nucleotide number 1 corresponding to the first base of the translation initiation codon.

Results

Clinical findings

Patients were classified as having either a CMD or LGMD phenotype and further subdivided according to the degree of structural and functional brain involvement. CMD was defined as onset of weakness prenatally or within the first 6 months of life. LGMD was defined by later onset weakness, specifically after having acquired ambulation. The cohort consisted of a total of 64 patients with CMD and 25 patients with LGMD, a total of 59 patients had brain involvement. In three cases the clinical information available was insufficient to determine phenotypic classification. Patients were divided into 1 of 7 broad phenotypic categories described below;

(1) WWS (and WWS-like): Onset prenatally or at birth. Patients assigned to this category had severe structural brain abnormalities including complete agyria or severe lissencephaly with only rudimentary cortical folding, marked hydrocephalus, severe cerebellar involvement and complete or partial absence of the corpus callosum. Eye abnormalities including congenital cataracts, micropthalmia and buphthalmus were common. When MRI evidence was not available, death before 1 year of age was taken as suggestive of this category if other clinical findings were supportive (Cormand *et al.*, 2001). Motor development was typically absent in these patients. Five patients were assigned to this group.
(2) MEB/FCMD-like: These categories were merged due to the overlapping phenotypic features. Included in

Table 1 Clinical characteristics of 33 individuals from 31 families in whom mutations were detected

Patient	ADG	Phenotype	Age at onset[a]	CK	Motor ability[b]	Contractures[c]	Hypertrophy[d]	Spine[e]	Eyes[f]	Weakness[g]	IQ[h]	Microcephaly[i]	MRI[j]	Other[k]
1	LOW	WWS	P	4000	NS	Y	Y	Sc, RS	Poor visual attention	LL>UL	L	Y	H, CHy, WM, Lis	Gastrostomy
2	LOW	MEB-FCMD	P	3500	N/A	Y	N/A	N/A	CG	N/A	L	Y	H, BS,WM, CC,CHy	N/A
3	LOW	LGMD-MR	I	2000	W	N/A	Y	U	N/A	N/A	L	Y	Normal	N/A
4	LOW	CMD-MR	I	7800	NW 2yr	N/A	N/A	U	U	N/A	L	N/A	WM	N/A
5	LOW	LGMD-MR	I	4000	W	N	Y	U	U	N/A	L	Y	Normal	N/A
6	LOW	LGMD-MR	3 Yr	8000	W		Y	N/A	U	N/A	L	Y	WM- minimal	N/A
7	LOW	CMD-MR	I	3600	St	N	Y	U	U	N/A	L	Y	Normal	N/A
8	LOW	CMD-MR	4 m	18000	W	N/A	Y	RS	N/A	N/A	L	Y	WM- minimal	Choreic Movement disorder
9	LOW	MEB-FCMD	N	5500	S	Y	Y	RS, Sc	N/A	N/A	L	Y	WM, BS	N/A
10	LOW	MEB-FCMD	4 Yr	5200	NW	N	Y	U		N/A	L	Y	Encephalocele	N/A
11	LOW	MEB-FCMD	7 m	N/A	NS	Y	N	U	Hm	N/A		N/A	H, WM,CC,	N/A
12	LOW	MEB-FCMD	N	3100	NS	Y	N/A	RS		UL>LL	L	Y	WM	N/A
13	N/A	MEB-FCMD	8 m		W	N/A	Y	U	My	UL>LL	L	N	WM, CDys, CC,PMG	N/A
14	LOW	MEB-FCMD	N	6000	S	Y	Y	Sc	CC	N/A	L	Y	BS,H,WM	SE, RIP age 11yr
15a	N/A	CMD-cerebellar	I	4700	W	Y	Y	N/A	N/A	UL>LL	L	Y	N/A	N/A
15b	N/A	CMD-cerebellar	I	5200	S	N/A	N/A	N/A	N/A	N/A	L	N/A	CHy	Micropenis and cryptorchidism
16	LOW	LGMD-MR	18 m	1900	W	N	Y	U	N/A	N/A	L	N/A	NO MRI	RBBB on ECHO
17	LOW	MEB-FCMD	N	2000	NS	Y	Y	N/A	My	UL,LL	L	Y	CHy, H	Macroglossia
18	LOW	MEB-FCMD	I	780	NW	N	N	N/A	CG	N/A	L	N/A	BS,CC,WM,H	N/A
19	LOW	MEB-FCMD	P	1000	W	Y	Y	U	OA, My	N/A	L	N	WM,CC	SE, feeding difficulties
20	LOW	LGMD-NOMR	12 Yr	12000	R	N	Y	U	My	LL>UL	N	N		N/A
21	LOW	MEB-FCMD	N	1200	NONE	N	N	U	RD	N/A	L	N/A	H,WM,CC	SE, feeding difficulties.

22	LOW	MEB-FCMD	12 m	2800	R	N	N/A	U	Pt, RA	N/A	L	N/A	CHy, CC, WM,H	Dyspraxia, feeding difficulties, SE
23	LOW	MEB-FCMD	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	H,CC,WM	N/A
24	N/A	WWS	N	1300	NS	N/A	N/A	U	N/A	N/A	L	N/A	CC,CHy, WM, H,Lis	N/A
25	LOW	WWS	P	5700	NONE	Y			RDy		L		H,WM,CHy, Lis	Feeding difficulties. RIP 8 weeks
26	LOW	CMD-NOMR	3 Yr	3200	S	N	N	U	U	G	N	N/A	WM-MILD	hypothyroid
27	N/A	MEB-FCMD	I	4000	S	N	Y	U	U	N/A	L	N/A	CC,WM,H	
28	LOW	WWS	N	7000	N/A	Y	Y	U	RD,Mo	N/A		N/A	WM,CHy, BS, H	Dysmorphic
29a	LOW	LGMD-NOMR	4 m	10000	W	N	Y	N/A	N/A	UL>LL	N	N	N/A	Steroid responsive
29b	LOW	LGMD-NOMR	4 m	13000	W	N	Y	U	U	LL>UL	N	N	Normal	Steroid responsive
30	LOW	LGMD-NOMR	10 m	60000	W	N	Y	U	U	LL>UL	N	N	H-MILD	Steroid responsive
31a	LOW	LGMD-NOMR	4 yr	9000	R	Y	N/A	U	N/A	N/A	N	N/A	N/A	N/A
32b	LOW	LGMD-NOMR	9 m	5700	R	Y	Y	U	N/A	LL>UL	N	N/A	Normal	CDH. Increased weakness with fever.

WWS = Walker–Warburg Syndrome; MEB/FCMD = Muscle-Eye-Brain/Fukuyama Congenital Muscular Dystrophy Like; CMD-MR = Congenital Muscular Dystrophy with Mental Retardation; CMD-NOMR = Congenital Muscular Dystrophy with No Mental Retardation; CMD-Cerebellar = Congenital Muscular Dystrophy with cerebellar Involvement; LGMD-MR = Limb Girdle Muscular Dystrophy with Mental Retardation; LGMD-NOMR = Limb Girdle Muscular Dystrophy with No Mental Retardation.
[a]P = prenatal onset; N = neonatal onset; I = infant onset; Yr = years; m = months. [b]W = walk; S = sit; St = stand; R = run; Prefix N = never. [c]Y = yes; N = no. [d]Y = yes; N = no. [e]RS = rigid spine; Sc = scoliosis; U = unaffected [f]CG = congenital glaucoma; RD = retinal detachment; RA = Retinal Atrophy; CC = Congenital cataracts; OA = optic atrophy; My = myopia; Mo = micropthalmia; Pt = ptosis; U = unaffected; Hm = hypermetropia; RDy = retinal dysplasia [g]UL = Upper limbs; LL = lower limbs; G = generalised [h]N = Normal intelligence; L = low [i]Y = yes; N = no [j]H = Hydrocephalus; CC = cerebellar cysts; BS = brainstem involvement; WM = white matter abnormality; CHy = cerebellar hypoplasia; Lis = lissencephaly; CDys = cerebellar dysplasia [k]SE = seizures; CDH = congenital dislocation of hip; RBBB = Right bundle branch block.

this group were CMD with brain abnormality less severe than that seen with WWS. MRI findings include pachygyria with preferential fronto-parietal involvement, polymicrogyria, cerebellar hypoplasia, cerebellar dysplasia and frequent flattening of the pons and brainstem. Eye abnormalities are often seen and include congenital glaucoma, progressive myopia, retinal atrophy and juvenile cataracts. Individuals may, rarely, acquire the ability to walk although this is delayed. Rarely patients manage to learn a few spoken words. Thirty patients were assigned to this group, including one in whom the clinical information was limited.

(3) CMD-CRB (CMD with cerebellar involvement): This category included CMD with mental retardation and cerebellar involvement on MRI scan as the only structural abnormality. Cerebellar abnormalities may include cysts, as described relatively frequently in individuals with *FKRP* gene defects (Mercuri *et al.*, 2006), or cerebellar hypoplasia or dysplasia. Four patients were assigned to this group.

(4) CMD-MR (CMD with mental retardation): CMD with mental retardation and structurally normal brain. Patients with isolated microcephaly or minor white matter changes on MRI are included in this group. Fifteen patients were assigned to this group, including two with limited clinical information.

(5) CMD-no MR (CMD with no mental retardation): Several patients within this group have had no neuroimaging but had entirely normal intellectual function. Ten patients were assigned to this group, one with limited information.

(6) LGMD-MR (LGMD with mental retardation): LGMD with mental retardation and structurally normal brain. Patients with minor white matter abnormalities and microcephaly were included in this group. This category includes patients with a phenotype resembling LGMD-2K (Balci *et al.*, 2005). Five patients were assigned to this group.

(7) LGMD-no MR (LGMD with no mental retardation): LGMD with no mental retardation. This category includes the LGMD phenotypes resembling LGMD2I and 2L (Godfrey *et al.*, 2006). Twenty patients were assigned to this group, six with limited clinical information.

The division of phenotypes within the cohort is shown in Table 4. Detailed clinical information is contained in Table 1 for those patients in whom pathogenic mutations were detected.

Mutation analysis

Mutation screening of *POMT1*, *POMT2*, *POMGnT1*, *fukutin* and *LARGE* was performed on 92 probands in whom *FKRP* mutations had been previously excluded. Homozygous and compound heterozygous mutations were detected in a total of 31 probands (34 individuals from 31 families). Thirty-seven different mutations were identified, 32 of which had not been previously reported. Pathogenic mutations are summarized in Table 2 and the comparative mutation frequencies between genes are represented in Table 4.

Without further RNA studies and functional biochemical analysis it is difficult to determine the pathogenicity of unclassified variants within these genes, this is exacerbated by the abundance of missense variants. For the purposes of this study, nonsense mutations, insertions and deletions, splice-site mutations as well as previously reported mutations were classified as pathogenic. Both exonic and intronic sequence alterations were categorized as polymorphisms if they were present on The Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov), the Leiden database (http://www.dmd.nl) or present as an additional change in a patient with two proven pathogenic mutations (polymorphisms are available in supplementary information, Table 2). Amino acid substitutions were classified as pathogenic if they were detected in conjunction with a clearly pathogenic mutation or if they have been shown to segregate with disease in a large pedigree. In addition, two patients with homozygous missense mutations and one patient with compound heterozygous missense mutations have been included in Table 2 as they are non-conservative amino acid changes that affected an evolutionary conserved amino acid residue (Patient 16, Patient 18 and Patient 27). Patients in whom only a single-sequence alteration was detected are summarized in Table 3. We have been unable to determine whether these are rare polymorphisms or pathogenic alterations in patients who harbor a second undetectable mutation. These six patients have not been included in the 34% of patients detected with mutations. Patient 25 has been included in Tables 1, 2 and 4 despite the absence of a second detectable mutation due to the presence of a nonsense mutation.

A variety of mutation types were identified; 37 missense mutations; 7 nonsense mutations; 9 frameshift mutations; 1 insertion/deletion mutation; 1 deletion and 6 splice-site mutations. No mutation hotspots were identified. From a total of 37 mutations, 8 were found to be recurrent within the cohort. The p.Ala200Pro mutation in *POMT1*, previously described as prevalent within the Turkish population (Balci *et al.*, 2005), was detected in three patients, two of Turkish origin and one of Greek decent (Patient 8). The *POMGnT1* donor splice-site mutation c.1539 + 1G>A found to account for the enrichment of MEB within the Finnish population was detected in two patients (Diesen *et al.*, 2004). Three further novel mutations were detected more than once, specifically the p.Tyr666Cys mutation which was found both in the homozygous and heterozygous state in four patients. Segregation of this novel missense mutation was studied in a large pedigree and was found to

Table 2 A summary of pathogenic mutations detected in this study

Patient	Gene	Exon/intron	Nucleotide change	Predicted amino acid change	Mutation type	Reference
1	*POMT1*	20	c.2179.2180delTC	p.Ser727fs	Frameshift	Novel
	POMT1	20	c.2179.2180delTC	p.Ser727fs	Frameshift	Novel
2	*POMT1*	20	c.2179.2180delTC	p.Ser727fs	Frameshift	Novel
	POMT1	20	c.2179.2180delTC	p.Ser727fs	Frameshift	Novel
3	*POMT1*	7	c.598G>C	p.Ala200Pro	Missense	Leiden database
	POMT1	7	c.598G>C	p.Ala200Pro	Missense	Leiden database
4	*POMT1*	18	c.1847.1849delGGT	p.Trp616del	Deletion	Novel
	POMT1	3	c.193G>A	p.Gly65Arg	Missene	Leiden database
5	*POMT1*	11	c.1081C>T	p.Gln361X	Nonsense	Novel
	POMT1	19	c.2005G>A	p.Ala669Thr	Missene	Novel
6	*POMT1*	6	c.517.523delTTCTTCAinsG	p.Phe173Asn175delinsAsp	Insertion/deletion	Novel
	POMT1	18	c.1868G>C	p.Arg623Thr	Missene	Novel
7	*POMT1*	7	c.598G>C	p.Ala200Pro	Missense	Leiden database
	POMT1	7	c.598G>C	p.Ala200Pro	Missense	Leiden database
8	*POMT1*	5	c.427G>T	p.Glu143X	Nonsense	Novel
	POMT1	7	c.598G>C	p.Ala200Pro	Missene	Leiden database
9	*POMT2*	21	c.2150T>C	p.Phe717Ser	Missense	Novel [a]
	POMT2	21	c.2177G>A	p.Gly726Glu	Missene	Leiden database
10	*POMT2*	19	c.1997A>G	p.Tyr666Cys	Missense	Novel
	POMT2	19	c.1997A>G	p.Tyr666Cys	Missense	Novel
11	*POMT2*	19	c.1997A>G	p.Tyr666Cys	Missense	Novel
	POMT2	11	c.1238G>C	p.Arg413Pro	Missene	Novel
12	*POMT2*	20	c.2047A>C	p.Thr683Pro	Missense	Novel
	POMT2	9	c.1051delG	p.Ala351fs	Framshift	Novel
13	*POMT2*	5	c.593T>A	p.Ile198Asn	Missense	Novel
	POMT2	19	c.1997A>G	p.Tyr666Cys	Missene	Novel
14	*POMT2*	10	c.1117G>T	p.Val373Phe	Missense	Novel
	POMT2	5	c.593T>A	p.Ile198Asn	Missene	Novel
15a, 15b [b]	*POMT2*	19	c.1997A>G	p.Tyr666Cys	Missense	Novel
	POMT2	19	c.1997A>G	p.Tyr666Cys	Missense	Novel
16	*POMT2*	5	c.551C>T	p.Thr184Met	Missense	Novel
	POMT2	21	c.2243G>C	p.Trp748Ser	Missene	Novel
17	*POMT2*	9	c.1057G>A	p.Gly353Ser	Missense	Novel[1]
	POMT2	21	c.2177G>A	p.Gly726Glu	Missene	Novel[1]
18	*POMGnT1*	6	c.526A>C	p.Thr176Pro	Missense	Novel
	POMGnT1	6	c.526A>C	p.Thr176Pro	Missense	Novel
19	*POMGnT1*	7	c.652+1G>A	Donor splice site	Splice site	Novel
	POMGnT1	17	c.1469G>A	p.Cys490Tyr	Missene	Leiden database
20 [b]	*POMGnT1*	20	c.1666G>A	p.Asp556Asn	Missense	Novel[2]
	POMGnT1	20	c.1666G>A	p.Asp556Asn	Missense	Novel[2]
21	*POMGnT1*	17	c.1539+1G>A	Donor splice site	Splice site	Leiden database
	POMGnT1	17	c.1539+1G>A	Donor splice site	splice site	Leiden database
22	*POMGnT1*	12	c.1100G>A	p.Arg367His	Missense	Novel
	POMGnT1	17	c.1539+1G>A	Donor splice site	Splice site	Leiden database
23	*POMGnT1*	20	c.1785+2T>G	Donor splice site	Splice site	Novel
	POMGnT1	20	c.1785+2T>G	Donor splice site	Splice site	Novel
24	*POMGnT1*	17	C1425G>A	p.Trp475X	Nonsense	Novel
	POMGnT1	17	C1425G>A	p.Trp475X	Nonsense	Novel
25	*LARGE*	13	c.1548C>G	p.Trp516X	Nonsense	Novel
26	*fukutin*	8	c.920G>A	p.Arg307Gln	Missense	Leiden database
	fukutin	8	c.920G>A	p.Arg307Gln	Missense	Leiden database
27	*fukutin*	8	c.915G>A	p.Trp305Cys	Missense	Novel
	fukutin	8	c.915G>A	p.Trp305Cys	Missense	Novel
28	*fukutin*	8	c.919C>T	p.Arg307X	Nonsense	Novel
	fukutin	8	c.919C>T	p.Arg307X	Nonsense	Novel
29a, 29b	*fukutin*	8	c.920G>A	p.Arg307Gln	Missense	Novel[3]

(continued)

Table 2 Continued

Patient	Gene	Exon/intron	Nucleotide change	Predicted amino acid change	Mutation type	Reference
	fukutin	9	c.1167dupA	p.Phe390fs	Frameshift	Leiden database[3]
30	fukutin	9	c.1167dupA	p.Phe390fs	Frameshift	Leiden database[3]
	fukutin	10	c.1363delG	p.Asp455fs	Frameshit	Novel[3]
31a, 31b	fukutin	4	c.340G>A	p.Ala114Thr	Missense	Novel
	fukutin	7	c.859delA	p.Thr286fs	Frameshift	Novel

Probands are numbered, affected siblings are indicated with letters.
[a]*de novo* mutation. [b]Family studies carried out to investigate segregation of the variant through the pedigree.
The following patients were included in this cohort and have recently been reported individually; [1]Patients previously described in Mercuri *et al.*, 2006. [2]Patient described individually in Clement *et al.*, 2007, *Archives of Neurology* in press. [3]Patients previously described in Godfrey *et al.*, 2006.

Table 3 Summary of unclassified variants

Patient	Gene	Exon/intron	Nucleotide change	Predicted amino acid change	Mutation type	Reference
32	*POMT1*	9	c.905T>G	p.Phe302Cys	missense	Novel
33	*POMT1*	19	c.1922C>T	p.Ala641Val	missense	Novel
34	*POMT1*	20	c.2203C>T	p.Arg735Cys	missense	Novel
35	*POMT1*	20	c.2244+5A>G	intronic	intronic	Novel
	POMT1	20	c.2244+5A>G	intronic	intronic	Novel
36	*POMT1*	20	c.2246G>A	synonymous	synonymous	Novel
37,38,39	*POMGnT1*	21	c.1867A>G	p.Met623Val	missense	Novel
39	*LARGE*	4	c.309C>A	synonymous	synonymous	Novel
40	*LARGE*	12	c.1431C>T	synonymous	synonymous	Novel
41	*LARGE*	13	c.1640G>A	p.Arg547His	missense	Novel
42,43	*LARGE*	14	c.1827A>T	synonymous	synonymous	Novel

Table 4 The phenotypic distribution of patients within the cohort, the frequency of mutations in each of the five glycosyltransferase genes analysed and the comparative mutation frequencies for individual clinical categories

	Number of patients							
	WWS	MEB FCMD	CMD CRB	CMD MR	CMD no MR	LGMD MR	LGMD no MR	Total
POMT1	1	1	–	3	–	3	–	8
POMT2	–	6	2	–	–	1	–	9
POMGnT1	–	6	–	–	–	–	1	7
fukutin	1	1	–	–	1	–	3	6
LARGE	1	–	–	–	–	–	–	1
Mutation detected	3 (60%)	14 (47%)	2 (50%)	3 (20%)	1 (10%)	4 (80%)	4 (20%)	31 (34%)
Total	5	30	4	15	10	5	20	92[a]

WWS = Walker–Warburg syndrome; MEB/FCMD = muscle eye brain syndrome/Fukuyama congenital muscular dystrophy; CMD CRB = congenital muscular dystrophy with cerebellar involvement; CMD-MR = congenital muscular dystrophy with mental retardation; CMD-no MR = congenital muscular dystrophy with no mental retardation; LGMD-MR = limb girdle muscular dystrophy with mental retardation; LGMD-no MR = limb girdle muscular dystrophy with no mental retardation.
[a]Includes three patients not assigned a clinical classification due to insufficient clinical information.

segregate with the disease (Patient 15). Parental samples were studied for 11 probands to ensure that compound heterozygous mutations were in *trans* and that apparent homozygous mutations in the proband were not masking undetected deletions. Where parental DNA was tested (22 families in total) a single paternal mutation was found to occur *de novo* (p.Phe117Ser, *POMT2*). A relatively similar frequency of patients with mutations were detected

in *POMT1*, *POMT2*, *POMGnT1* and *fukutin* (Table 4). In contrast, only a single patient was found to have a pathogenic mutation in *LARGE* although we were unable to identify a second mutation (Patient 25).

Genotype–phenotype correlations

The spectrum of phenotypes associated with mutations in *POMT1* included WWS (one case), MEB-FCMD (one case), CMD-MR (three cases) and LGMD-MR (three cases). *POMT2* mutations were observed in patients with MEB-FCMD (six cases), CMD-CRB (two cases) and LGMD-MR. Six patients with *POMGnT1* mutations had WWS and a single case had LGMD-no MR. Phenotypes associated with mutations in *fukutin* were detected in patients with WWS (one case), MEB-FCMD (one case), CMD-no MR (one case) and LGMD-no MR (three cases). A mutation in *LARGE* was detected in a single patient with WWS.

Although α-DG immunostaining was not systematically quantified as part of this study, we noticed a broad correlation between the amount of depleted glycosylated epitope and phenotypic severity. For example the WWS patient found to have a mutation in *LARGE* had complete absence in immunostaining, while the previously reported milder case of MDC1D had only a reduction in the amount of immunofluorescence (Longman *et al.*, 2003). Similarly the *POMGnT1* patient with LGMD-no MR (Patient 20) had only a subtle deficiency in immunofluorescence (Clement *et al.*, 2007, *Archives of Neurology*, in press), in contrast to the virtually absent expression in patients with MEB-FCMD.

There was no clear difference in phenotype or pattern of dystroglycan expression between patients with and without mutations in any of these genes. The phenotypic spectrum of patients without identifiable mutations was similar to that of patients with mutations.

Discussion

Dystroglycanopathies are a recently defined, common group of muscular dystrophies encompassing an extremely wide spectrum of clinical severity and are caused by mutations in at least sex genes encoding putative or demonstrated glycosyltransferases. The comparatively small coding region of *FKRP* has facilitated the rapid correlation between genotype and phenotype, allowing the discovery of pathogenic mutations in patients with LGMD2I, MDC1C, MEB-like and WWS-like disorders. However, there is no information regarding the frequency of involvement or the genotype–phenotype relationships for the remaining five glycosyltransferase genes in a large and unbiased population.

In this study we have systematically screened for mutations in *POMT1*, *POMT2*, *POMGnT1*, *fukutin* and *LARGE* in patients in whom we had previously ruled out *FKRP* gene involvement. Mutations were detected in 34% of these patients.

Fukutin mutations

Mutations in *fukutin*, typically associated with FCMD in Japan were found in six patients, none of whom are of Japanese origin. Only two of these patients had structural brain involvement; one patient affected by WWS (Patient 28) and one by a MEB-FCMD phenotype (Patient 27). The remaining patients had no structural brain involvement; one case had CMD-no MR (Patient 26) and never acquired the ability to walk but has normal IQ and five individuals from three families have entirely normal intellect and a mild LGMD phenotype (LGMD2L) (Patients 29, 30 and 31). Interestingly in the latter two of these families, a dramatic response to steroid therapy was noted (Godfrey *et al.*, 2006). In striking contrast to what has previously been reported in FCMD, none of these five patients have evidence of central nervous system involvement. Our findings together with the recent description of individuals with *fukutin* mutations presenting with a predominant cardiomyopathy (Murakami *et al.*, 2006), suggest that the majority of mutations outside Japan give rise to conditions milder than FCMD and are not usually associated with structural brain involvement.

POMGnT1 mutations

Mutations in *POMGnT1* were also associated with a wider than reported spectrum of clinical severity, which include a relatively mild form of LGMD. The majority of patients (6) had an MEB like disorder with only a single patient possessing a LGMD phenotype, suggesting that *POMGnT1* mutations more frequently give rise to congenital disorders with associated structural brain involvement. The LGMD patient (Patient 20) has entirely normal intellectual function and disease onset in the second decade of life, dramatically expanding the phenotypes associated with mutations in *POMGnT1* (Clement *et al.*, 2007, *Archives of Neurology*, in press).

POMT1 mutations

Mutations in *POMT1* have previously been reported in patients with WWS, CMD-MR and LGMD-MR (LGMD-2K). Within our cohort, all patients with mutations in *POMT1* had evidence of functional brain involvement either with no clear associated structural brain abnormalities (three patients with LGMD2K, and three patients with CMD-MR), or more severe conditions with structural brain defects (one patient with WWS, and one individual with a MEB-like phenotype). This suggests that the majority, if not all patients with *POMT1* mutations have either functional or structural central nervous system involvement, including those patients with relatively mild muscle weakness. This is in contrast to patients in the present study with mutations in *fukutin* and *POMGnT1* and to that previously reported for *FKRP* mutations.

POMT2 mutations

Mutations in *POMT2* were confined to patients with evidence of brain involvement. Nine patients had pathogenic *POMT2* mutations; six with a MEB-FCMD phenotype, two with a CMD-cerebellar phenotype and a single patient with LGMD-MR. This latter patient has learning difficulties and remains ambulant at age 20 having presented, at 18 months of age, with developmental delay (Patient 16). These findings indicate that like *POMT1*, the majority or all patients with mutations in *POMT2* have evidence of central nervous system involvement. In addition, we have identified the mildest phenotype associated with mutations in *POMT2* reported to date in an individual with LGMD-MR.

LARGE mutations

We were only able to identify a single pathogenic *LARGE* mutation in a patient with typical WWS phenotype who died in the first few months of life (Patient 25). Absent immunofluorescence staining was demonstrated on this patient's skeletal muscle biopsy using antibodies which recognise the glycosylated epitope of α-DG. Unfortunately neither sufficient DNA nor frozen muscle from this patient was available to investigate the presence of a second, as yet undetected, mutation. However, it remains possible that this mutation contributed to the patient's phenotype.

Mutation frequencies

Mutations in *POMT2* were the most prevalent with nine cases, followed by *POMT1* with eight cases, *POMGnT1* with seven cases, *fukutin* with six cases and finally *LARGE* with only a single case.

We have previously identified *FKRP* mutations in 79 patients. Approximately 75% of these patients have a LGMD2I phenotype (Brockington *et al.*, 2001*a*; Topaloglu *et al.*, 2003; Beltran-Valero de Bernabe *et al.*, 2004; Mercuri *et al.*, 2006). The relative frequency of *FKRP* involvement needs to be considered with caution as it clearly reflects the genetic origin of patients studied in our unit. For example screening of 79 Australian LGMD patients detected only two *FKRP* mutations. However, when amalgamating these results, it remains clear that *FKRP* mutations are the most frequently found mutations in this group of conditions. Both ourselves and others have previously published extensively on the spectrum of these mutations (Brockington *et al.*, 2001*a*, *b*; Mercuri *et al.*, 2003; Topaloglu *et al.*, 2003; Harel *et al.*, 2004; Mercuri *et al.*, 2006; Vieira *et al.*, 2006; Lin *et al.*, 2007).

Genotype–phenotype correlations

Pathogenic mutations were detected in 3 of 5 patients with WWS syndrome (60%), 14 of 30 patients with a MEB/FCMD phenotype (47%), 2 of 4 patients with CMD CRB (50%), 3 of 15 patients with CMD-MR (20%), 1 of 10 patients with CMD-no MR (10%), 4 of 5 patients with LGMD-MR (80%) and 4 of 20 patients with LGMD-no MR (20%) (Table 4).

Patients with associated structural brain defects belonging to the severe end of the clinical spectrum showed no apparent difference in their pattern of skeletal muscle weakness or central nervous system involvement in relation to the gene involved. However, the four LGMD patients with associated MR and microcephaly all had mutations in either *POMT1* or *POMT2*. No mutations were identified in the remaining patients. Conversely a number of patients with more severe muscle weakness and no brain involvement (CMD-no MR) were found to have mutations in *fukutin*, similar to that previously described in MDC1C (Brockington *et al.*, 2001*a*). This suggests that there may be a hierarchical involvement of muscle and brain arising from individual gene mutations, with *POMT1* and *POMT2* being associated with significant central nervous system involvement even in patients with relatively mild weakness and who remain ambulant (LGMD2K). This does not appear to be a feature of *fukutin* or *FKRP*. These results suggest that in some individual subcategories, certain genes are more likely to be involved than others and this should be taken into account when undertaking mutation analysis in the dystroglycanopathies.

The results of this study demonstrate that the phenotypic spectrum of disorders associated with mutations in the six known glycosyltransferase genes is significantly wider than initially suspected, in part due to the high prevalence of founder mutations within specific populations (Kobayashi *et al.*, 1998; Diesen *et al.*, 2004). We have expanded the clinical phenotypes associated with mutations in *POMT1*, *POMT2*, *POMGnT1*, *fukutin* and *LARGE*, although we have not observed a full spectrum of phenotypes associated with each gene, in particular *POMT1*, *POMT2* and *LARGE*. A large number of patients with clinico-pathological features indistinguishable from the ones detailed in this manuscript were not found to have mutations in any of the genes studied. Finally, this work suggests that more, as yet undefined, genes are likely to be involved in the pathogenesis of the dystroglycanopathies. The identification of these genes may provide additional information on the pathway of glycosylation of α-dystroglycan.

Supplementary material

Supplementary material is available at *Brain* online.

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References

Akasaka-Manya K, Manya H, Kobayashi K, Toda T, Endo T. Structure-function analysis of human protein O-linked mannose beta1,2-N-acetylglucosaminyltransferase 1, POMGnT1. Biochem Biophys Res Commun 2004; 320: 39–44.

Akasaka-Manya K, Manya H, Nakajima A, Kawakita M, Endo T. Physical and functional association of human protein O-mannosyltransferases 1 and 2. J Biol Chem 2006; 281: 19339–45.

Balci B, Uyanik G, Dincer P, Gross C, Willer T, Talim B, et al. An autosomal recessive limb girdle muscular dystrophy (LGMD2) with mild mental retardation is allelic to Walker-Warburg syndrome (WWS) caused by a mutation in the POMT1 gene. Neuromuscul Disord 2005; 15: 271–5.

Barresi R, Campbell KP. Dystroglycan: from biosynthesis to pathogenesis of human disease. J Cell Sci 2006; 119: 199–207.

Beltran-Valero de Bernabe D, Currier S, Steinbrecher A, Celli J, van Beusekom E, van der Zwaag B, et al. Mutations in the O-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker-Warburg syndrome. Am J Hum Genet 2002; 71: 1033–43.

Beltran-Valero de Bernabe D, Voit T, Longman C, Steinbrecher A, Straub V, Yuva Y, et al. Mutations in the FKRP gene can cause muscle-eye-brain disease and Walker-Warburg syndrome. J Med Genet 2004; 41: e61.

Brockington M, Blake DJ, Prandini P, Brown SC, Torelli S, Benson MA, et al. Mutations in the fukutin-related protein gene (FKRP) cause a form of congenital muscular dystrophy with secondary laminin alpha2 deficiency and abnormal glycosylation of alpha-dystroglycan. Am J Hum Genet 2001*a*; 69: 1198–209.

Brockington M, Muntoni F. The modulation of skeletal muscle glycosylation as a potential therapeutic intervention in muscular dystrophies. Acta Myol 2005; 24: 217–21.

Brockington M, Torelli S, Prandini P, Boito C, Dolatshad NF, Longman C, et al. Localization and functional analysis of the LARGE family of glycosyltransferases: significance for muscular dystrophy. Hum Mol Genet 2005; 14: 657–65.

Brockington M, Yuva Y, Prandini P, Brown SC, Torelli S, Benson MA, et al. Mutations in the fukutin-related protein gene (FKRP) identify limb girdle muscular dystrophy 2I as a milder allelic variant of congenital muscular dystrophy MDC1C. Hum Mol Genet 2001*b*; 10: 2851–9.

Brown SC, Torelli S, Brockington M, Yuva Y, Jimenez C, Feng L, et al. Abnormalities in alpha-dystroglycan expression in MDC1C and LGMD2I muscular dystrophies. Am J Pathol 2004; 164: 727–37.

Cormand B, Pihko H, Bayes M, Valanne L, Santavuori P, Talim B, et al. Clinical and genetic distinction between Walker-Warburg syndrome and muscle-eye-brain disease. Neurology 2001; 56: 1059–69.

Currier SC, Lee CK, Chang BS, Bodell AL, Pai GS, Job L, et al. Mutations in POMT1 are found in a minority of patients with Walker-Warburg syndrome. Am J Med Genet A 2005; 133: 53–7.

de Bernabe DB, van Bokhoven H, van Beusekom E, Van den Akker W, Kant S, Dobyns WB, et al. A homozygous nonsense mutation in the fukutin gene causes a Walker-Warburg syndrome phenotype. J Med Genet 2003; 40: 845–8.

de Paula F, Vieira N, Starling A, Yamamoto LU, Lima B, de Cassia Pavanello R, et al. Asymptomatic carriers for homozygous novel mutations in the FKRP gene: the other end of the spectrum. Eur J Hum Genet 2003; 11: 923–30.

Diesen C, Saarinen A, Pihko H, Rosenlew C, Cormand B, Dobyns WB, et al. POMGnT1 mutation and phenotypic spectrum in muscle-eye-brain disease. J Med Genet 2004; 41: e115.

Dubowitz V, Sewry CA. Muscle biopsy - a practical approach: [Saunders]; 2007.

Godfrey C, Escolar D, Brockington M, Clement EM, Mein R, Jimenez-Mallebrera C, et al. Fukutin gene mutations in steroid-responsive limb girdle muscular dystrophy. Ann Neurol 2006; 60: 603–10.

Harel T, Goldberg Y, Shalev SA, Chervinski I, Ofir R, Birk OS. Limb-girdle muscular dystrophy 2I: phenotypic variability within a large consanguineous Bedouin family associated with a novel FKRP mutation. Eur J Hum Genet 2004; 12: 38–43.

Kobayashi K, Nakahori Y, Miyake M, Matsumura K, Kondo-Iida E, Nomura Y, et al. An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. Nature 1998; 394: 388–92.

Lin YC, Murakami T, Hayashi YK, Nishino I, Nonaka I, Yuo CY, et al. A novel FKRP gene mutation in a Taiwanese patient with limb-girdle muscular dystrophy 2I. Brain Dev 2007; 29: 234–8.

Longman C, Brockington M, Torelli S, Jimenez-Mallebrera C, Kennedy C, Khalil N, et al. Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of alpha-dystroglycan. Hum Mol Genet 2003; 12: 2853–61.

Manya H, Sakai K, Kobayashi K, Taniguchi K, Kawakita M, Toda T, et al. Loss-of-function of an N-acetylglucosaminyltransferase, POMGnT1, in muscle-eye-brain disease. Biochem Biophys Res Commun 2003; 306: 93–7.

Mercuri E, Brockington M, Straub V, Quijano-Roy S, Yuva Y, Herrmann R, et al. Phenotypic spectrum associated with mutations in the fukutin-related protein gene. Ann Neurol 2003; 53: 537–42.

Mercuri E, Topaloglu H, Brockington M, Berardinelli A, Pichiecchio A, Santorelli F, et al. Spectrum of brain changes in patients with congenital muscular dystrophy and FKRP gene mutations. Arch Neurol 2006; 63: 251–7.

Muntoni F, Brockington M, Blake DJ, Torelli S, Brown SC. Defective glycosylation in muscular dystrophy. Lancet 2002; 360: 1419–21.

Murakami T, Hayashi YK, Noguchi S, Ogawa M, Nonaka I, Tanabe Y, et al. Fukutin gene mutations cause dilated cardiomyopathy with minimal muscle weakness. Ann Neurol 2006; 60: 597–602.

Toda T, Kobayashi K, Takeda S, Sasaki J, Kurahashi H, Kano H, et al. Fukuyama-type congenital muscular dystrophy (FCMD) and alpha-dystroglycanopathy. Congenit Anom (Kyoto) 2003; 43: 97–104.

Topaloglu H, Brockington M, Yuva Y, Talim B, Haliloglu G, Blake D, et al. FKRP gene mutations cause congenital muscular dystrophy, mental retardation, and cerebellar cysts. Neurology 2003; 60: 988–92.

van Reeuwijk J, Brunner HG, van Bokhoven H. Glyc-O-genetics of Walker-Warburg syndrome. Clin Genet 2005a; 67: 281–9.

van Reeuwijk J, Janssen M, van den Elzen C, Beltran-Valero de Bernabe D, Sabatelli P, Merlini L, et al. POMT2 mutations cause alpha-dystroglycan hypoglycosylation and Walker-Warburg syndrome. J Med Genet 2005b; 42: 907–12.

Vieira NM, Schlesinger D, de Paula F, Vainzof M. Mutation analysis in the FKRP gene provides an explanation for a rare cause of intrafamilial clinical variability in LGMD2I. Neuromuscul Disord 2006; 16: 870–3.

Williamson RA, Henry MD, Daniels KJ, Hrstka RF, Lee JC, Sunada Y, et al. Dystroglycan is essential for early embryonic development: disruption of Reichert's membrane in Dag 1-null mice. Hum Mol Genet 1997; 6: 831–41.

Xiong H, Kobayashi K, Tachikawa M, Manya H, Takeda S, Chiyonobu T, et al. Molecular interaction between fukutin and POMGnT1 in the glycosylation pathway of alpha-dystroglycan. Biochem Biophys Res Commun 2006; 350: 935–41.

Yoshida A, Kobayashi K, Manya H, Taniguchi K, Kano H, Mizuno M, et al. Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. Dev Cell 2001; 1: 717–24.

Mild POMGNT1 Mutations underlie a Novel Limb-Girdle Muscular Dystrophy Variant
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